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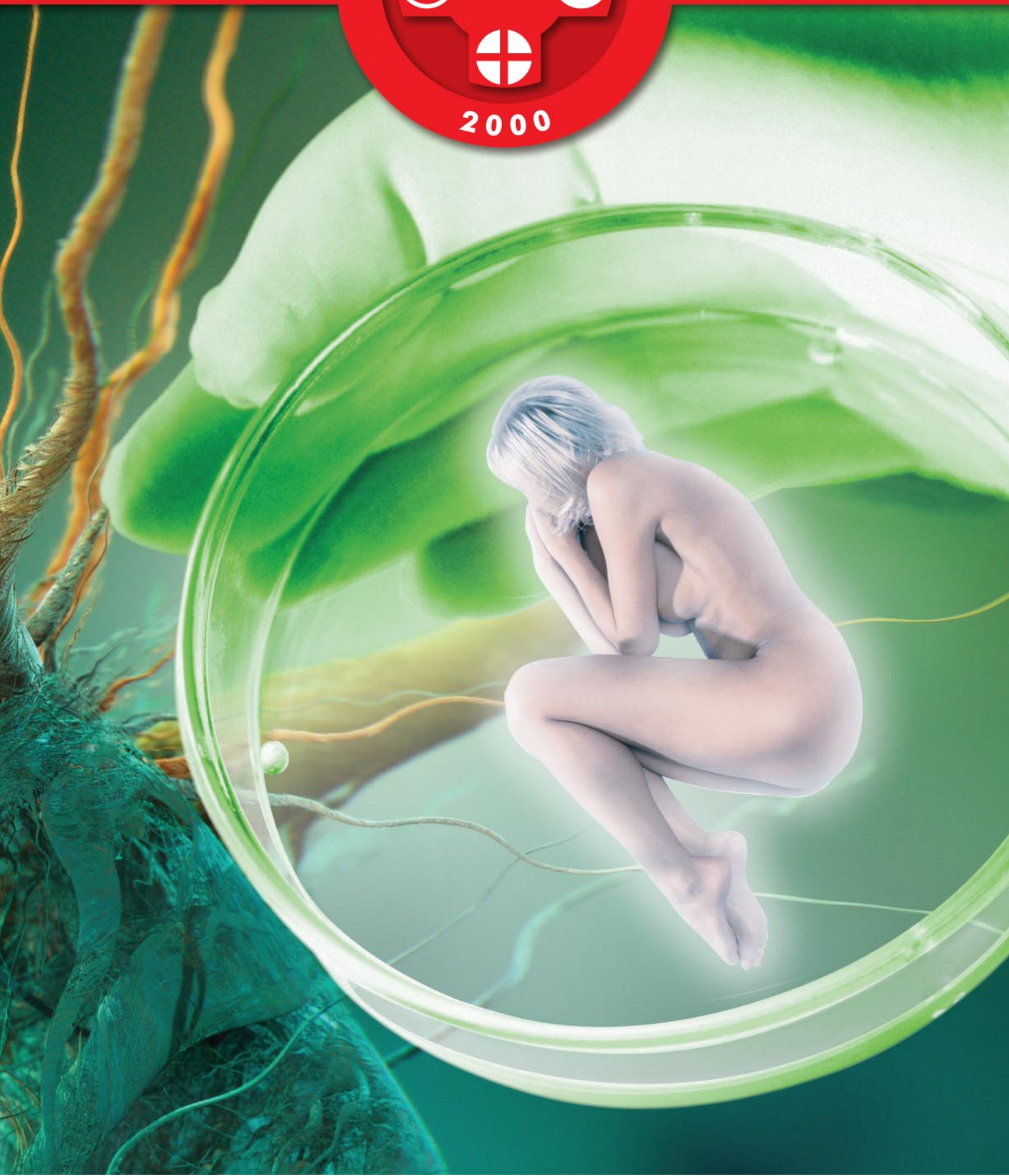

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Svetozara Markovića 69, 34000 Kragujevac, PO Box 124
Serbia
e-mail: sjecr@medf.kg.ac.rs
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POSITIVE EFFECT OF HUMAN ESC CONDITIONED MEDIUM ON SOMATIC CELL REPROGRAMMING

Katarzyna Tilgner [1,2], Lyle Armstrong [1,2] and Majlinda Lako [1,2]
[1] Centro de Investigacion Principe Felipe, Valencia, Spain
[2] Institute of Human Genetics, Newcastle University, United Kingdom

ABSTRACT

Induced pluripotent stem cells (iPSC), created by reprogramming of somatic cells into embryonic-like state are the latest developments in stem-cell research. These cells have a great potential in research and medicine, however the full exploitation of these cells is hampered by several issues such as derivation efficiency, heterogeneity and safety. Here we describe a simple method to enhance generation of fully reprogrammed human iPSCs from dermal fibroblasts. We have shown that the addition of culture medium that has been previously conditionned by human embryonic stem cells (hESC-CM) at the final stage of reprogramming procedure (≥3rd week) improves the efficiency of somatic cell reprogramming, possibly by promoting the transition of pre-iPSC colonies to a fully reprogrammed state. This approach may offer an alternative to the epigenetic modulators and chemicals method in the generation of safer and more efficient iPSCs.

Introduction Several pieces of research including the cloning of Dolly the sheep (Wilmut et al., 1997), put to rest for once and for all a long believed dogma that the cellular patterning of cells during the mammalian embryogenesis is irreversible. This break-through was accomplished due to iPSC technology, a method by which any somatic cell type may be reprogrammed in vitro to a pluripotent-like state by the introduction of specific transcription factors and subsequent culture under ESC-like conditions. Cells generated in such way, referred to as induced pluripotent stem cells (iPSCs), have been proven to be similar to embryonic stem cells in terms of morphology, gene expression and differentiation abilities. Such characteristics have raised hopes for the generation of patient specific iPSCs for biomedical research and clinical applications. Though, before any personalized stem cell-based therapies can be considered, a number of limitations needs to be addressed. The risk of potential mutagenesis due to the genomic insertion of exogenous reprogramming factors has been partially overcomed in recent years by replacing integrating retro- and lentiviruses with transiently transgene-expressing carriers (such as inducible/excisable vectors, adenoviruses), however the slow kinetics coupled with very low efficiency (≤0.01%) (Takahashi et al., 2007, Yu et al., 2007) and heterogeneous nature of emerging iPSC colonies are still significant handicaps for iPSC technology. As part of the undergoing effort to solve these problems we found that hESC-culture medium conditioned with hESC (hESC-CM) improves the efficiency of somatic cell reprogramming possibly by promoting the transition of pre-iPSC colonies to a fully reprogrammed state.

MATERIALS AND METHODS

Lentiviral transduction and reprogramming culture: Lentiviral vectors for human OCT4, NANOG, LIN28 and SOX2 were obtained from Stemgent (iPSC Generation Human TF Lentivirus Set; Cat. No.00-005). Lentiviral transductions of neontal human fibroblasts (NHDF, Lonza) were carried out (at an M.O.I of 5) with cells in attachment (1x105 cells/2ml/well of 6-well plate seeded the day before transduction) in fibroblasts medium in the presence of polybrene (0.6 μg/ml final concentration, Sigma). Following the overnight incubation with lentiviral transduction mixture, human somatic cells were dissasociated with trypsin and transferred to 6-well plate seeded with Mitomicin C inactivated MEFs (from 1 well of 6-well plate transduced cells to 3 wells of 6-well MEF feeder plate). Twenty-four hours later the fibroblasts medium was replaced with hESC culture media in which cells were maintained for one week. After this time the transduced cells were either cultured (1) continuously in human ESC culture medium conditioned with inactivated MEF (F-CM) or (2) starting from day 21- in human ESC medium conditioned with inactivated MEF (F-CM) supplemented 1:1 with hESC culture medium collected from cultivated hESCs (referred here as human ESC medium conditioned with hESC (ESC-CM). ESC-CM medium was collected from H9 cells 3-5 days after plating and prior use was filtered through 0.2 μm filter to avoid cross-contamination. In both strategies media were refreshed every day until colonies with a similar morphology to hESCs were observed, which was usually between 28-35 days after transduction. Once iPSC colonies were identi-

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Correspondence to: Majlinda Lako, Centro de Investigacion Principe Felipe
Avda. Autopista del saler, 16-3 Junto Oceanografico 46012 Valencia Spain,
tel:+34 9632 89681 ext. 1211, mlako@cipf.es

fied, they were manually picked and serially expanded on Mitomycin C inactivated MEF feeder plates in conventional hESC growth conditions for several passages.

Immunocytochemistry: ALP staining was performed according to the manufacturer's instructions using the Alkaline Phosphatase Detection Kit (Milipore). For pluripotency markers staining, cells were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) and washed with PBS. Additionally for internal marker staining the cells were incubated in permabilisation solution (0.2% Triton X-100 (Sigma-Aldrich) in PBS) for 10 min at RT. They were then incubated for 30 min in blocking buffer (5% goat serum and 1% BSA in PBS). Afterwards the primary antibodies (mouse anti -OCT4 1:100 (Millipore); mouse anti-TRA-1-60 1:100 (Millipore) and mouse anti-SSEA-4 1:100 (BD Pharmingen), were applied in blocking buffer for 1 hour at RT. Then the cells were washed with PBS and incubated with secondary antibodies (anti-mouse IgG FITC conjugated 1:500 (Sigma)) in blocking buffer for 45 min at RT in dark. Nuclei were detected by DAPI (Sigma-Aldrich) staining. Images were captured using a Xiovert software and a Zeiss microscope.

RESULTS

Generation of induced pluripotent stem cells by defined factors is greatly improved by medium conditioned with hESC (ESC-CM)

To induce reprogramming, human dermal fibroblast cells (NHDF) were co-transfected with a set of viruses coding genes known to be critical for maintenance of pluripotency in embryonic stem cells (OCT4, SOX2, NANOG and LIN28). Transduced cells were then cultured under conditions specified in materials and methods (see also Figure 1A) and the reprogramming progress was monitored on a daily basis by epifluorescence microscopy. As presented in Figure 1B morphological changes were observed as early as day 4 post-transduction. At this time some fibroblast appeared spherical in their morphology and formed initially loose followed by densely packed clusters. These early iPSC colonies, displaying a granulated non-human ES cell-like morphology, became dominant in early reprogramming days as they proliferated actively, however at the second week post-transduction the majority of them disappeared. Since it has been demonstrated by others that it is at ~10-12 post-transduction when the switch from the viral transgene expression to endogenenous gene activation takes place (Stadtfeld et al., 2008), it is very likely that most of our early iPSC colonies did not pass this crucial step for further reprogramming and possibly reverted back into fibroblast or simply died. Colonies which displayed the appropriate hESCs morphology (Figure 1B) emerged much later (≥day 28) and interestingly their number differed significantly between different culture conditions (Figure 1C). When OSLN transduced cells were maintained from day 7 up to the end (35 days) in F-CM, only a few colonies appeared (average of 3±1 colonies per 1x105 cells initially used). However, when transduced cells from day 21 were treated with F-CM in combination with hESC-CM (at 1:1 ratio), a significant increase in the number of colonies that closely resembled the hESC morphology was observed (20±2 colonies per 1x105cells) (Figure 1C). In both cases the colonies with good morphology, i.e. tightly packed, flat and with defined edges, were manually picked out for expansion and identity analyses. The immunocytochemistry assay (Figure 1D), showed that selected clones, regardless of the culture strategy used, exhibited strong Alkaline Phosphatase (AP) activity and were positive for the pluripotency marker-specific cell surface (TRA-1-60, TRA-1-81, SSEA-4) and nuclear (OCT4, SOX2) markers. Further molecular examination for the expression of pluripotency and differentiation markers, bisulphite genomic sequencing analyses of certain promoters together with more sophisticated in vitro and in vivo analyses will further dissect how closely newly generated iPSCs resemble hESCs and whether the hESC-CM supplement has affected the quality of reprogrammed iPSC clones.

Discussion Conventionally human ESCs are maintained in culture in undifferentiated state either on inactivated feeder layer in serum-free medium or as feeder-free in the presence of conditioned medium produced by inactivated feeders. Several studies on conditioned medium have shown that secreted factors produced by fibroblasts are important for hESC cultivation (Lim and Bodnar, 2002; Xie et al., 2004; Chin et al., 2007). It is therefore not surprising that conditioned medium has been utilised the majority of current reprogramming procedures, including ours. By culturing OSLN transduced fibroblast in conditioned media we indeed observed a large number of potential iPSC colonies emerging at early days of reprogramming, however only a very small number of them made into the final stage ultimately giving rise to fully reprogrammed iPSCs. This is why we decided to further modify the reprogramming procedure by supplementing the conditioned media collected from feeders (F-CM) with conditioned media collected from hESC cultures (hESC-CM). We speculated that hESCs may secrete to the media some factors which may support their maintenance (positive loop) and accordingly may help to gain pluripotent state by cells undergoing reprogramming. In our study, treatment of OSLN transduced cells with hESC conditioned medium (starting from day 21) reproducibly enhanced the reprogramming efficiency at least 10 times, possible by promoting the transition of pre-IPSC colonies to a full reprogrammed state. This observation encourages more proteomic analyses on hESC-conditioned media. Such screens may lead to the identification of molecules that will become powerful tools in providing us with new insights into the reprogramming process and may ultimately lead to an efficient iPSC generation protocol.

ACKNOWLEDGMENTS:

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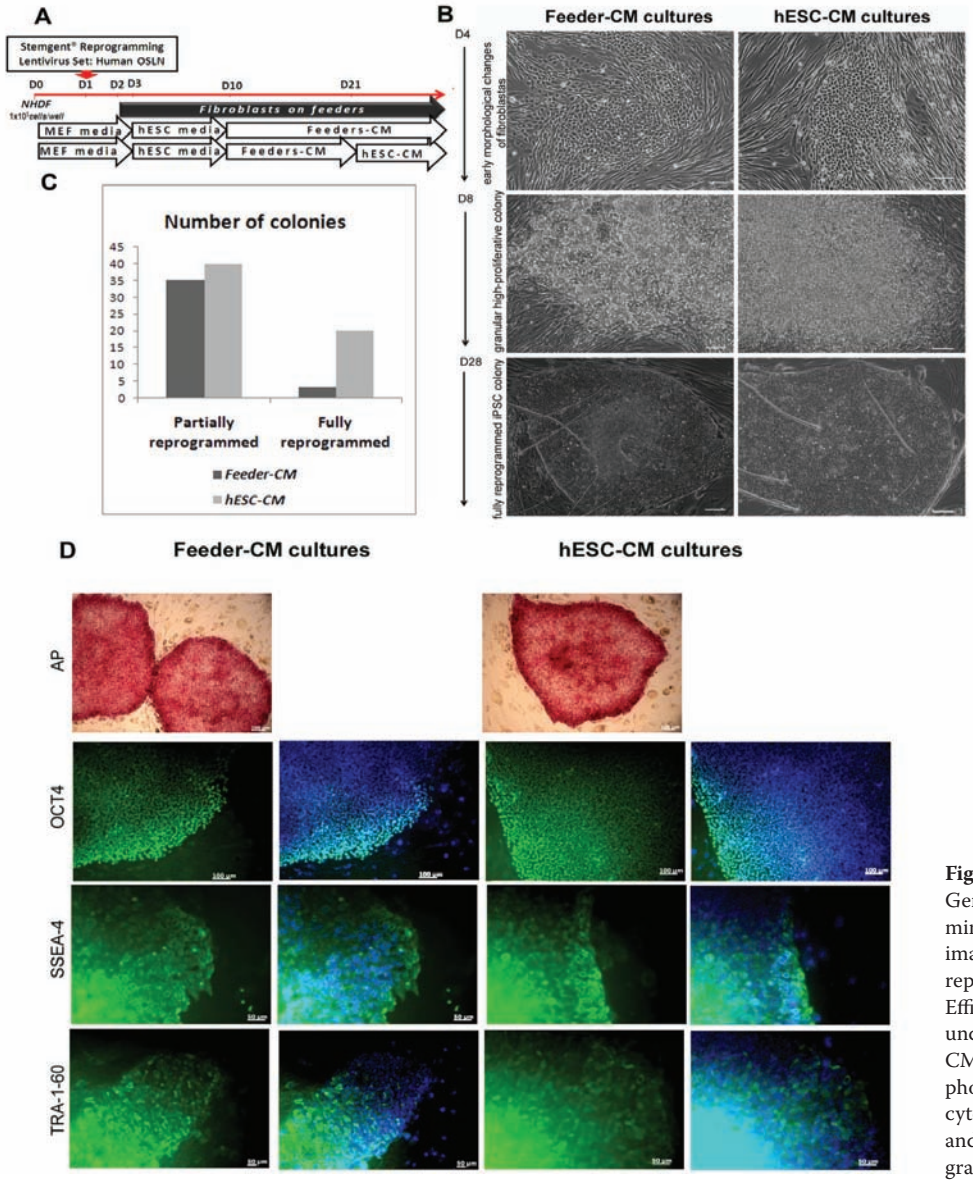

Figure 1
General outline of the reprogramming procedure (A); Microscope images of early, partially- and fully reprogrammed iPSC colonies (B); Efficiency of colony formation under F-CM and F-CM –hESC-CM (1:1) conditions (C); Alkaline phophatase staining and immunocytochemistry of OCT4, SSEA-4 and TRA-1-60 on fully reprogrammed colonies (D).

Th-17 CELLS AS NOVEL PARTICIPANTS IN IMMUNITY TO BREAST CANCER

Ivan Jovanovic[1], Gordana Radosavljevic[1], Sladjana Pavlovic[1], Nemanja Zdravkovic[1], Katerina Martinova[1], Milan Knezevic[1], Danijela Zivic[2], Miodrag L. Lukic[1] and Nebojsa Arsenijevic[1]
[1] Center for Molecular Medicine, Faculty of Medicine, University of Kragujevac, Serbia
[2] Clinical Center Kragujevac, Serbia

TH-17 LIMFOCITI, NOVI UČESNIK U IMUNSKOM ODGOVORU NA TUMOR DOJKE

Ivan Jovanović[1], Gordana Radosavljević[1], Sladjana Pavlović[1], Nemanja Zdravković[1], Katerina Martinova[1], Milan Knežević[1], Danijela Živić[2], Miodrag L. Lukić[1] and Nebojša Arsenijević[1]
[1] Centar za Molekulsku Medicinu, Medicinski Fakultet, Univerzitet u Kragujevcu, Srbija
[2] Klinički Centar Kragujevac, Srbija

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ABSTRACT

*Breast cancer is a leading cause of cancer-related deaths among women worldwide. Tumour surveillance constitutes a process of recognising and modifying tumour development and involves both innate and adaptive immune systems. During the progression of malignancy, the immune response is dynamically changed. In our breast cancer model, we used 4T1 mouse mammary tumour cell lines with the capacity to metastasise efficiently to sites affected by human breast cancer. This model was used to evaluate anti-tumour immunity and tested **in vivo** whether tumour progression affected anti-tumour immunity. Female BALB/c mice were injected with 5 x 10^4 4T1 tumour cells into 4-th mammary fat-pad. Tumour size was evaluated daily and the number and size of tumour metastases was determined on day 36. Serum levels of pro-inflammatory cytokines, leukocyte cytotoxicity and cellular make up of the draining lymph nodes were tested in animals on day 13 after tumour inoculation. On day 36, metastases were found in the lungs and livers of the mice. IL-17 levels were higher in tumour bearing mice compared to healthy animals, while TNF-α serum levels showed no significant differences during tumour progression. Total cellularity of the draining lymph nodes was higher in tumour bearing mice. There were no differences in the total number of CD8+ and CD4+ cells; however, significant increases in CD19+ cells were found on the 13th day after tumour inoculation. Finally, MTT tests indicated higher cytotoxic activity levels in the draining lymph node cells of tumour bearing mice. We provide evidence suggesting that tumour induction may enhance immune responses most likely via the enhancement of Th-17 cells and the attenuation of CD4+Foxp3+ Treg cells.*

Key words: *mouse breast cancer, 4T1, metastasis, Th-17, Treg*

SAŽETAK

Tumor dojke je vodeći uzrok smrti kod žena širom sveta. Imunski nadzor predstavlja proces prepoznavanja i eliminacije malignih ćelija, koji uključuje i urođenu i stečenu imunost. Tokom progresije tumora, imunski sistem trpi dinamične promene. U ovom eksperimentu koristili smo 4T1 ćelijsku liniju mišjeg tumora dojke kao model tumora koji daje metastaze u organima zahvaćenim kod humanog karcinoma dojke. Cilj našeg istraživanja bio je ispitati efekte progresije tumora na anti-tumorsku imunost kod eksperimentalnog modela karcinoma dojke na BALB/C miševima. BALB/C miševima ženskog pola ubrizgano je 5 x 10^4 4T1 tumorskih ćelija direktno u masno jastuče mlečne žlezde broj 4. Veličina primarnog tumora merena je svakodnevno, a broj i veličina metastatskih kolonija 36-og dana erksperimenta. Trinaestog dana od ubrizgavanja tumorskih ćelija merili smo serumske nivoe pro-inflamatornih citokina, citotoksičnost leukocita i ćelijski sastav drenirajućih limfnih čvorova. Tridesetšestog dana od indukcije tumora, nadjene su metastaske kolonije na plućima i jetri. Izmeren je znatno viši serumski nivo IL-17 u miševima sa tumorima, a nije nadjena značajna promena u nivou TNF-α tokom progresije bolesti. Ukupna celularnost drenirajućih limfnih čvorova je povećana, 13-og dana nakon indukcije tumora. Nije pronadjena razlika u ukupnom broju CD8+ i CD4+ limfocita, ali je registrovano značajno povećanje ukupnog broja CD19+ limfocita. Ukupan broj CD4+Foxp3+ limfocita je značajno smanjen istog dana eksperimenta. Konačno, izmerena je veća citotoksična aktivnost tumor drenirajućih limfocita. Na osnovu navedenih rezultata, mi smatramo da indukcija tumora može da facilitira imunski odgovor, najverovatnije kroz aktivaciju Th-17 limfocita i smanjenje broja CD4+Foxp3+ T regulatornih limfocita (Treg).

Ključne reči: *mišji tumor dojke, 4T1, metastaze, Th-17, Treg*

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Correspondence to: Ivan Jovanovic, Medical Faculty, University in Kragujevac, Serbia,
tel. +381 34 306 800, ivanjovanovic77@gmail.com

INTRODUCTION

Breast cancer is a leading cause of cancer-related deaths among women worldwide. Breast cancer genesis is caused, in part, by a combination of oncogenic mutations that promote genetic instability and accelerated cellular proliferation (1). The major cause of mortality from breast cancer is due to metastasis to distant organs, such as the lungs, bones, liver and brain (2). Breast cancer does not induce potent and effective immune responses (3). However, tumour surveillance constitutes a process of recognising and modifying tumour development and involves both innate and adaptive immune systems (4).

Detection of T lymphocytes in carcinoma tissue has revealed that they are associated with tumour development. The important role of T cells as effectors in anti-tumour immunity was first shown in numerous experimental models. For instance, UV light-induced tumours have been shown to grow progressively in the absence of T cells and are normally rejected by normal mice (5-7). The mature T-cell population is composed of 1) αβ T cells expressing CD4 or CD8 and 2) CD4-/CD8- γδ T-cell receptor (TCR)-expressing cells.

Most tumours are positive for MHC class I and negative for MHC class II, and CD8+ T cells are able to induce tumour killing upon direct recognition of peptide antigens, which are presented by the tumour's MHC class I molecules (8).

CD4+ T cells (T-helper lymphocytes, Th) can also recognise tumour antigens either directly or via cross-presentation by host antigen presenting cells (8). Th cells, as an integral part of adaptive immunity, have a bipolar role in mounting anti-tumour responses. The CD4+ T cell population can be divided into two subpopulations based on types of cytokine secretion (9). Type 1 Th cells characteristically secrete IFN-g, whereas type 2 T cells secrete IL-4, IL-5, IL-10, and IL-13. The commitment of CD4+ T cells to either a type 1 or type 2 pathway is influenced by many factors, including the nature of antigen (10), costimulatory molecules (11), the type of antigen-presenting cells and the cytokine environment (12, 13). Th1 and Th2 cells play important immunoregulatory roles in cancer development (14). There have been many reports suggesting that the Th1-type anti-tumour immune response provides a greater therapeutic impact. The role of Th1 cells in anti-tumour response is often to aid in the activation of CD8+ T cells. On the other hand, Th2-type cytokines usually downregulate anti-tumour immunity, although they can promote the recruitment of tumouricidal eosinophils and macrophages into the tumour microenvironment (15-18).

B lymphocites present contributors to the anti-cancer immune response via the secretion of antigen-specific immunoglobulins. They likewise facilitate the recruitment of innate leukocytes and the targeted destruction of neoplastic cells (19). Besides the role of B cells in tumour regression through immunoglobulin-mediated mechanisms, recent data are also pointing to a potential role in tumour development.

Interleukin-17 (IL-17), T cell-derived cytokine, was originally described as cytotoxic T lymphocyte (CTL)-associated antigen 8 (20). Interleukin 17 is predominantly produced by activated CD4 T-cells, but some studies in humans have demonstrated that CD8 T-cells can also produce IL-17 (21). It is considered to be a proinflammatory cytokine because it increases IL-6 and IL-8 production by macrophages, fibroblasts, keratinocytes, and synovial cells (22-26) and also induces the secretion of IL-1b and TNF-α by human macrophages and endothelial cells (24, 27). TNF-α was originally identified for its capacity to induce hemorrhagic necrosis of solid tumours (29). Its anti-tumour effects work both through direct cytotoxicity against tumour cells, but also through the activation of macrophages, cytotoxic lymphocites and neutrophils (30, 31), as well as specific damage to tumour blood vessels (32-34). IL-17 and TNF-α represent pleiotropic cytokines that are critical to multiple biological processes and exert a great influence on the development, progression and immune surveillance of tumours.

Regulatory T cells (Treg) represent a subset of CD4+T cells that function to modulate immune responses through the ability to suppress T-cell proliferation and cytokine production (35). The majority of Treg lymphocytes express high levels of interleukin-2 (IL-2) receptor α chain (CD25) and transcription factor Foxp3. These cells constitute 2-3% of CD4+ human blood T cells. Tregs have considerable influence on the regulation of immune response in autoimmunity but also play an important role in cancer development.

In the current study, we developed a breast cancer model using a 4T1 mouse mammary tumour cell line with the capacity to metastasise efficiently to sites affected in human breast cancer to evaluate the role of Th-17 cells in a particular tumour model.

MATERIALS AND METHODS

Animals

Female BALB/c mice (obtained from the Military Medical Academy), aged 8 to 9 weeks, were used in the experiments. Mice were housed under standard conditions. The experiments were approved by the ethics board of the Medical Faculty of Kragujevac.

Tumour cells

The weakly immunogenic mouse breast tumour cell line 4T1, which is singenic to the BALB/c background, was purchased from the American Type Culture Collection (Manassas, USA). The tumour cell line was derived from a single spontaneously arising mammary tumour from a BALB/C mouse (36). The rapid and efficient metastasis to organs affected in human breast cancer makes the 4T1 model an excellent mouse model for the study of the progression of breast cancer in humans. 4T1 cells were maintained in DMEM supplemented with 10% FBS, 2 mmol/l L-glutamine, 1 mmol/l penicillin-streptomycin and 1

mmol/l mixed nonessential amino acids (PAA Laboratories GmbH), a complete growth medium. Subconfluent monolayers in log growth phase were harvested by brief trypsin treatment, using 0,25% trypsin and 0,02% EDTA in PBS (PAA Laboratories GmbH) and washed three times in serum-free PBS before use in all in vitro and in vivo experiments. The number of viable tumour cells was determined by the trypan blue, and only those cell suspensions with more than 95% viable cells were used.

Induction of tumour

Syngenic female BALB/c mice were injected with 50 µl of a single-cell suspension containing 5×10^4 4T1 mammary carcinoma cells, orthotopically into the fourth mammary fat-pad of mice (direct injection). The size of the primary tumour in diameter was daily assessed morphometrically using electronic callipers and is presented as the mean ± SEM. Mice were sacrificed on the 13th and 36th days after tumour cell injection, and the primary tumours were surgically removed. Blood (from the mice's abdominal aortas), and samples of lungs, liver, brain and sentinel lymph nodes were collected. For the purposes of the study, the sentinel lymph node was defined as the primary draining lymph node for the primary cancer (37). Specimens of lungs, liver and brain were routinely embedded in paraffin, stained with hematoxylin and eosin (H&E) and reviewed to confirm the presence of metastatic colonies. Tumour cells appeared heterogeneous in size but were easily differentiated from non-tumour cells as predominately larger cells with an elevated nuclear to cytoplasm ratio. To avoid missing micrometastases, 4 µm H&E-stained sections from at least three different levels were examined for the presence of metastases. The number and size of metastatic colonies were examined with light microscopy by an independent observer.

Measurement of cytokines

Sera from animals were collected by a single needle stick and were stored at –20 °C until thawed for assay. Serum levels of IL-17 and TNFα were measured in one sample with highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN) specifically receptive to the mouse cytokines. In brief, premixed standards were reconstituted in PBS (pH 7.2), generating a stock concentration of 2000 pg/mL for TNF-α and 1000 pg/mL for IL-17. The standard stocks were serially diluted in Reagent Diluent to generate 7 points for the standard curves. Diluted Capture Antibody was added in a 96-well, flat-bottomed, polystyrene microtiter plate (MTP), with a final volume of 100µl. The plates were sealed and incubated overnight at room temperature and then washed with Wash Buffer (autowasher). The samples were diluted 1:4 in the Reagent Diluent. Premixed standards or diluted samples (100 µl) were added to each well containing washed beads, and then were covered with an adhesive strip and incubated for 2 hours at room temperature. After incubation and washing, 100 µL of the premixed Detection Antibody was added to each well, and then the wells were covered with a new adhesive strip and incubated for 2 hours at room temperature. After incubation and washing, Streptavidin-HRP was added to each well (100 µL). The incubation was terminated after 20 min. at room temperature (avoiding placement of the plates in direct light). After washing, the beads were then re-suspended in 100 µl of Substrate Solution. Then, 50 µL of Stop Solution were added to each well, and optical density of each well was immediately determined using a microplate reader set to 450 nm.

Cell preparation

Thirteen days after injection with the tumour cells, the mice were sacrificed, and their sentinel (inguinal) lymph nodes were isolated. Further, single-cell suspensions from the sentinel lymph nodes were obtained by mechanical dispersion through steel and nylon mesh screens in complete growth medium. After three washes, the cells were re-suspended in complete growth medium.

Cytotoxicity assay

To examine cytotoxic activity, we divided the mice into two groups: mice injected with tumour cells and healthy mice. Cells isolated from sentinel lymph nodes were used as effector cells in this assay. 4T1 mouse breast tumour cells were used as targets. The target cells were plated in 96-well flat bottom plates at a density of $1x10^4$ cells/well (V= 100 µl) in growth medium, in triplicate. After culture at 37°C for 24 h, effector cells were added at $4x10^4$ cells/well (V= 100 µl) to yield a target:effector (T:E) ratio of 1:4. After co-culture at 37°C for 24 h, methylthiazolyldiphenyl-tetrazolium (MTT; Sigma Chemical, St. Louis, MO) was added to each well for a final concentration of 5 mg/ml. Four hours later, the plates were centrifuged at 1000 rpm for 5 min, the medium was gently removed, MTT crystals were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO), and the optical density was read on a spectrophotometer (OD570). The percentage of cytotoxity was calculated as: cytotoxity (%) = [1 - (experimental group (OD)/control group (OD))] x 100 (38). Data are expressed as the mean of triplicate wells ± SEM.

Flow cytometry

To investigate whether the administration of breast cancer cells could affect the number of lymphocytes derived from draining lymph nodes, the number of lymphocytes was measured using flow cytometric analysis scan (FACS). Single-cell suspensions of sentinel lymph nodes were obtained from mice on day 13 after the tumour cells were injected. Cells (5 x 105/ml) were washed three times and re-suspended in cold PBS containing 0.1% sodium azide (Sigma) and 10% mouse serum. Subsequently, they were incubated with FITC- or PE-labelled mAbs specific for mouse CD4, CD8, CD25, CD19 and F4/80 (BD Pharmingen, USA) or isotype-matched controls (5 mg/ml), for 30 min at 4°C in PBS.

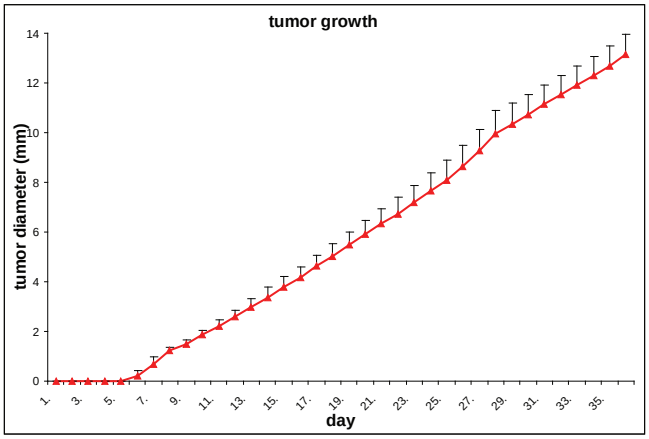
For the analysis of regular T cells, we used double staining. After labelling surface marker CD4, we conducted an intracellular staining technique for detecting Foxp3. CD4-labelled cells were washed in cold PBS. Cell pellets were then re-suspended using pulse vortex in 1 ml of freshly prepared fixation/permeability working solution and incubated for 2 hours in the dark. They were washed once by adding 2 ml of permeabilisation buffer followed by centrifugation and decanting of the supernatant. The washing procedure was then repeated. Then, the cells were incubated in the dark in 100 µl of Fc block in permeabilisation buffer for 30 min at 4°C. After blocking and without washing, PE-labelled anti-Foxp3 antibody (BD Pharmingen, USA) was added to the suspension, and the mixture incubated in the dark for 30 min at 4°C. As a control, we used an isotype control in the permeabilisation buffer instead of fluorochrome. The cells were then washed in 2 ml of permeabilisation buffer and centrifuged before the supernatant was decanted.

Stained cells were analysed by FACS calibre flow cytometry (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson). Dead cells were excluded by gating out propidium iodide-positive cells.

number of mice with metastases/ total number of mice		
lungs	liver	brain
6/7	4/7	0/7

Table 1.
The incidence of metastases in mice inoculated with 4T1 tumour cells

1A)



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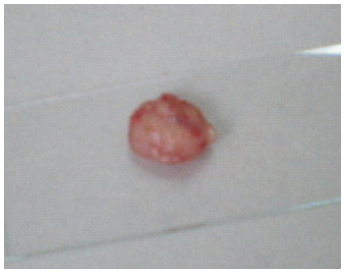
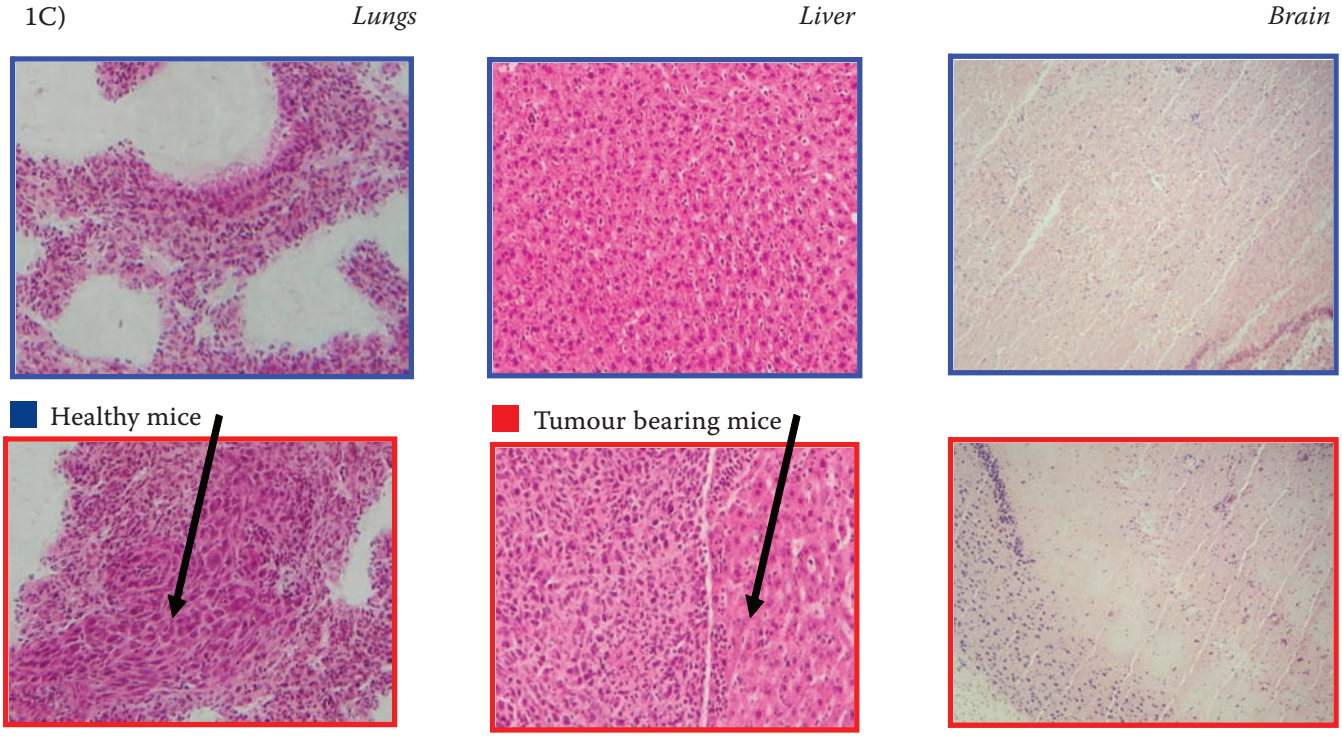


Figure 1:

A. Mean values of tumour diameters in BALB/C mice at 36 days after inoculation of 5x104 4T1 cells per mouse. On the 36th day of the experiment, the mean value of primary tumour diameters was 13,16 ± 0,79 mm.
B. Picture of surgically removed primary tumour.
C. Light-microscopic pictures of sections through pulmonary, liver and brain tissue (arrows are pointing on metastatic colonies).

1C)



Statistical analysis

For statistical analysis, the two-tailed Student's t-test or nonparametric Mann–Whitney Rank Sum test was used. The data were analysed using the SPSS statistical package, version 13.

RESULTS

1. Detection of tumour growth and metastasis

The primary tumour was established in the BALB/c mice by a unilateral subcutaneous injection of 5×10^4 4T1 mammary carcinoma cells, orthotopically into the fourth mammary fat-pad. Tumour growth was measured daily, using callipers, as described in the materials and methods sections. The results pertaining to tumour growth and metastasis are shown in **Figure 1** and **Table 1**. Systemic tumour involvement was determined by microscopic assessment. Specimens of lungs, liver and brain were investigated for the presence of metastatic colonies. Metastasis became apparent 5 to 6 weeks after tumour inoculation, although metastasising cells had probably seeded these sites earlier (77,78). Six out of seven BALB/C mice (86%) developed numerous lung metastatic colonies, while four out of seven (57%) developed lung metastases, as shown in Table 1. No brain metastases were detected.

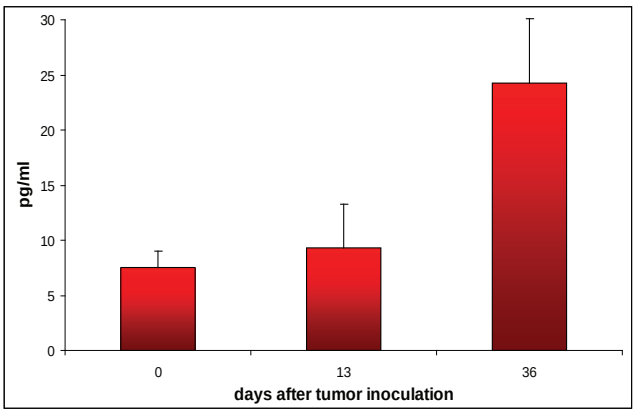
2. Serum levels of proinflammatory cytokines after tumour inoculation

To assess the anti-tumour immune response, we investigated the systemic production of proinflammatory cytokines. The measurements were performed before and on days 13 and 36 after tumour inoculation. After tumour inoculation, we noticed an increase in IL-17, and on the 36th day of the experiment, increases became significant when compared to baseline (24,21 ± 5,89 vs. 7,54 ± 1,45), as shown in **Figure 2A** (p=0.047). At the same time, we found the opposite trend in TNF-α serum levels. That is, TNF-α levels showed evident, but not significant, decreases during tumour progression, as shown in **Figure 2B.**

3. Anti-tumour cytotoxicity

To investigate the anti-tumour immune response, we analysed the cytotoxicity of sentinel lymph node cells. BALB/C mice were inoculated subcutaneously with 5×10^4 4T1 breast tumour cells orthotopically into the fourth mammary fat-pad. On day 13, 4T1-treated and equivalent untreated mice were killed, and their sentinel (inguinal) lymph nodes were removed. Lymph node cell suspensions were prepared and $4x10^4$ cells were plated into 96-well flat bottom plates and pre-incubated with $1x10^4$ 4T1 cells to yield a target:effector (T:E) ratio of 1:4. The percentage of cytotoxicity was determined after 24 hours of culture. Cells from tumour bearing mice manifested significantly higher cytotoxic activity compared with untreated BALB/C mice (57,13 ± 1,11 vs. 39,83 ± 1,47 %; p=0.027), as shown in **Figure 3.**

2A)



2B)

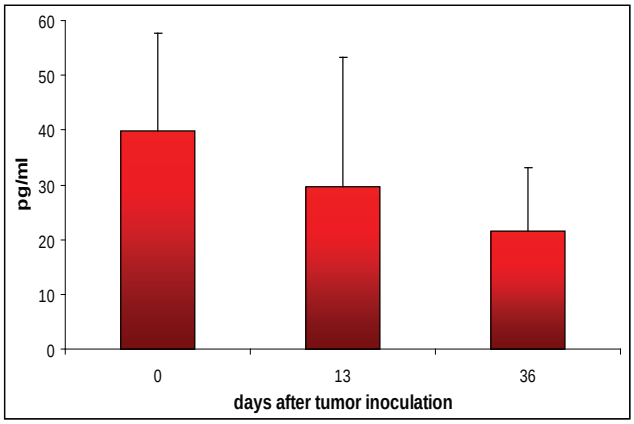


Figure 2:

A. Serum levels of IL-17 in BALB/C mice at 0, 13 and 36 days after inoculation of 5x104 4T1 cells per mouse. Serum levels of IL-17 were higher in tumour bearing mice 36 days after inoculation when compared with healthy mice (24,21 ± 5,89 vs. 7,54 ± 1,45; p=0.047).

B. Serum level of TNF-α in BALB/C mice at 0, 13 and 36 days after inoculation of 5x104 4T1 cells per mouse. Serum levels of TNF-α were lower in tumour bearing mice 36 days after inoculation when compared with the healthy animals (21,52 ± 11,69 vs. 39,93 ± 17,77).

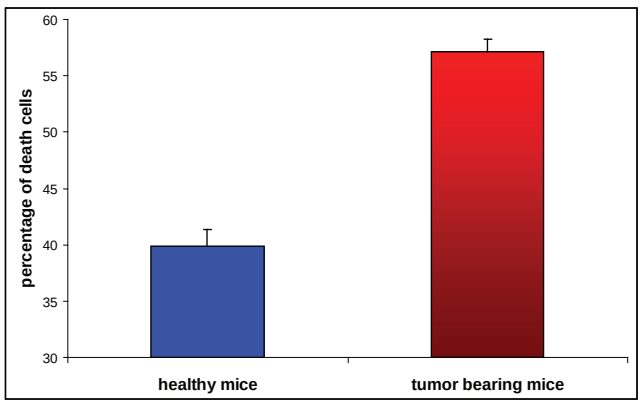
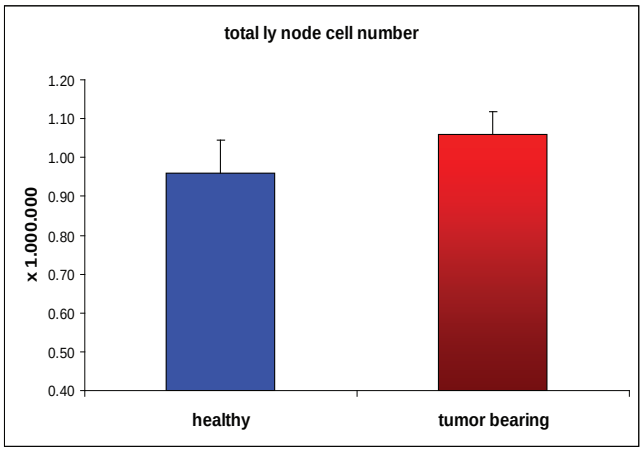
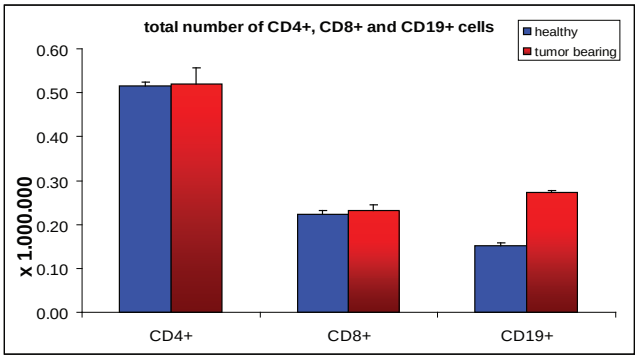


Figure 3. Cytotoxity of leukocytes derived from tumour draining lymph nodes 13 days after the injection of 5x104 4T1 tumour cells and from the inguinal nodes from healthy animals. When compared with the healthy mice (39,83 ± 1,47), the percentage of cytotoxity of leukocytes from tumour bearing mice was higher (57,13 ± 1,11; p=0.027).

4A)



4B)



4C)

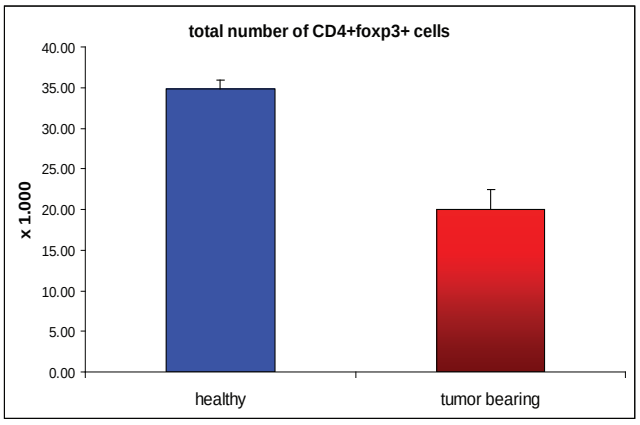


Figure 4. FACS analysis of lymph node derived leukocytes in BALB/C mice, before and after tumour inoculation:

A. The total lymph node cell number. Results showed no significant increases after tumour injection (0,96 ± 0,08 x 106 vs. 1,06 ± 0,06 x 106 cells).

B. Total number of different T and B cell populations. After tumour inoculation, no significant changes were found in the number of CD+ cells (515.225 ± 8.351 vs. 519.448 ± 37.866) or CD8+ cells (223.887 ± 7.647 vs. 232.290 ± 12.742). However, the number of B lymphocytes significantly increased after the tumour injection (150.890 ± 6.170 vs. 273.820 ± 2.380).

C. Total number of CD4+Foxp3+ cells in the draining lymph nodes. The number of CD4+Foxp3+ cells decreased after tumour inoculation (34.830 ± 1.040 vs. 20.040 ± 2.470).

4. Cellular composition of lymphoid cells in sentinel nodes (day 13)

To assess and characterise the cellular make up of sentinel nodes and their possible correlations with disease progression, sentinel lymph nodes were extirpated on 13th day after tumour inoculation, and lymphocyte populations were enumerated by multicolour flow cytometric analysis. As shown in **Figure 4A**, the results suggest that there is an slight increase in the number of total cells in the draining lymph nodes after tumour inoculation (0,96 ± 0,08 x 106 vs. 1,06 ± 0,06 x 106 cells; p>0.05). The same trend was evaluated in CD4+ (515.225 ± 8.351 vs. 519.448 ± 37.866) and CD8+ T cell populations (223.887 ± 7.647 vs. 232.290 ± 12.742). The number of CD19+ cells (B- lymphocytes) derived from inguinal lymph nodes showed significant increases after tumour injection (150.890 ± 6.170 vs. 273.820 ± 2.380), as shown in **Figures 4B** and 5E-F (p=0.001). Furthermore, it appears that the number of CD4+Foxp3+ cells was decreased during tumour progression (34.830 ± 1.040 vs. 20.040 ± 2.470), as shown in **Figures 4C** and 5G-H (p=0.054).

DISCUSSION

The 4T1 mammary carcinoma cell line was originally isolated by Fred Miller and his colleagues at the Karmanos Cancer Institute (39). We introduced this weakly immunogenic mouse breast tumour orthotopically into the mammary fat pad of the animals. The tumours grew rapidly at the primary site and formed metastases in the lungs, liver, bone and brain over a period of 3-6 weeks. Its use has increased in recent years because of its high propensity to metastasise to bone and other sites (40). Because this model is syngenic in BALB/c mice, we are using it to study the role of the immune system in tumour progression. In the current study, we showed rapid tumour growth, reflected through primary tumour diameters. On day 36 after the inoculation of tumour cells, 4T1 tumour cells had spread into different anatomical locations. The lungs, liver and brain from mice killed at day 36 were recovered. Visible metastases were found in the lungs and liver.

During the progression of malignancy, immune responses changed dynamically. When studying the inflammatory responses against tumours, we discovered a higher expression of the pro-inflammatory cytokine IL-17 on the 36th day after tumour inoculation as compared to baseline levels. This hints at a role of IL-17 in the inflammatory response to breast cancer progression. Interleukin 17 is predominantly produced by activated CD4 T-cells (41). CD4+ T cells can be classified into T-helper (Th) 1 cells, which secrete interferon (IFN) γ, IL-2, and tumour necrosis factor (TNF), and β and Th2 cells, which produce IL-4, IL-5, IL-6, IL-10, and IL-13, Additionally, there are also Th0 cells, a common precursor with the ability to release both IFNg and IL-4 (42). Thirty percent of Th0/Th1 clones have been shown to produce IL-17, whereas Th2 clones never express IL-17 (41). However, there is consensus now that IL-17 and IL-22 producing cells represent separate Th-

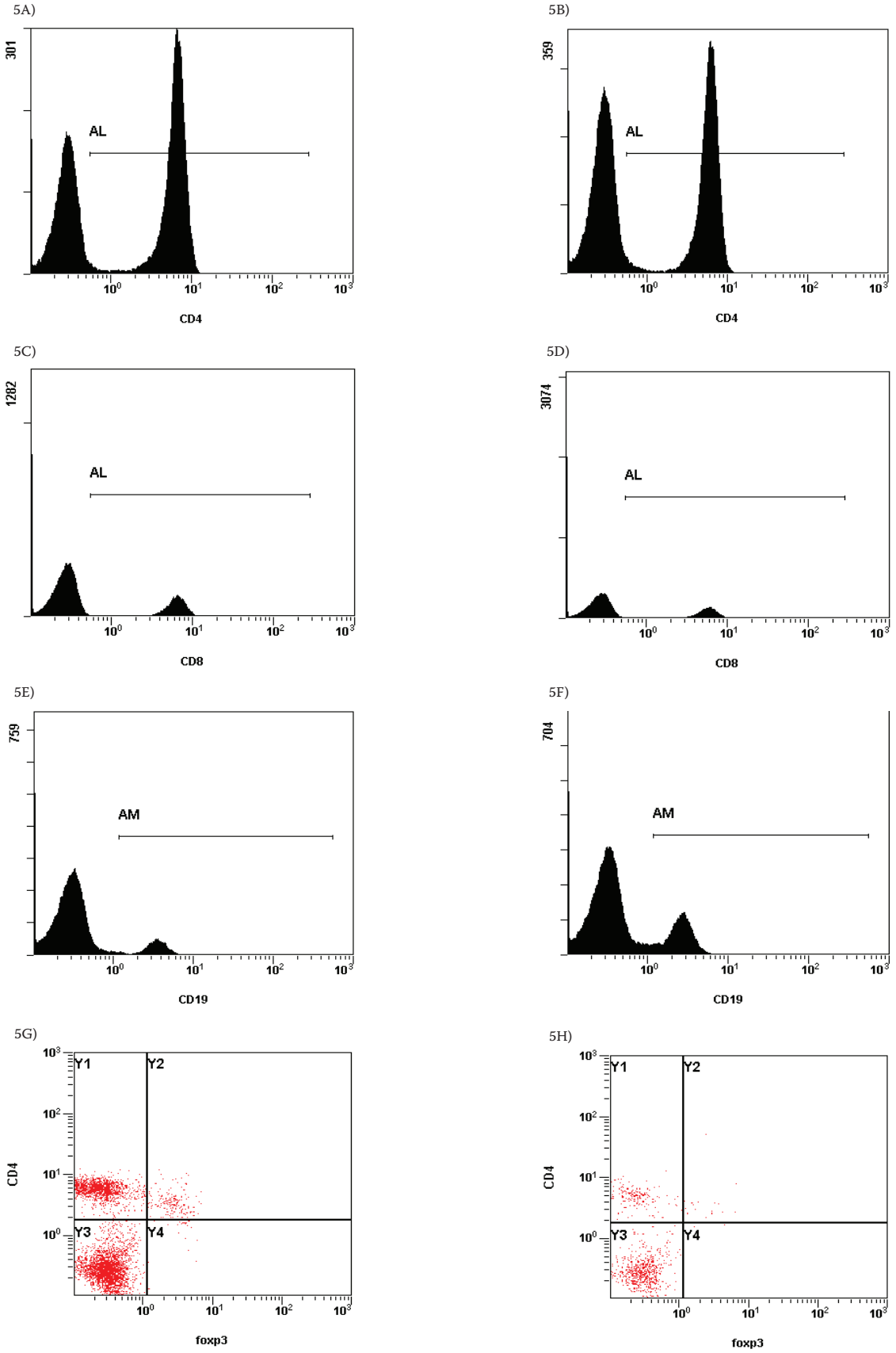


Figure 5. FACS analysis of lymph node derived leukocytes in BALB/C mice, before and after tumour inoculation:
A. CD4+ cells in healthy mice; B. CD4+ cells in tumour bearing mice; C. CD8+ cells in healthy mice; D. CD8+ cells in tumour bearing mice; E. CD19+ cells in healthy mice; F. CD19+ cells in tumour bearing mice; G. CD4+foxp3+ cells in healthy mice; H. CD4+foxp3 cells in tumour bearing mice.

17 cell populations. IL-17 is a pro-inflammatory cytokine because it increases IL-6, IL-8, IL-1b and TNF-α by many different cell populations (22-27). IL-17 is upregulated in breast cancer (28) and can influence tumour progression in a dual manner. IL-17 can inhibit the growth rate of tumours through the enhancement of tumour-specific T-cell activity (42). It has been shown that IL-17 increases the production of IL-6 by different cells (43), which is associated with the induction of tumour-specific CTLs (44, 45). It is also known (24) that IL-17 stimulates the secretion of IL-12 by macrophages, promoting Th1 immunity, and leads to the activation of CTLs (46). Additionally, IL-17 promotes breast cancer invasion (47), through upregulation of the metalloproteinases MMP-2 and MMP-9 (48), which indicates a pro-tumour effect of inflammation.

It was understood that TNF-α had a critical role in chronic inflammatory diseases such as rheumatoid arthritis (49), but it appears that it also plays a role in tumour progression. For many years, TNF-α was thought to have only anti-tumour effects (29, 30-34), but recent studies are demonstrating its tumour-promoting role (50-55). We found no significant changes in TNF-α serum levels during tumour progression.

The purpose of the next study phase was to characterise and quantify cells that are involved in the anti-tumour immune response in sentinel lymph nodes 13 days after tumour induction as well as compare them with control lymph nodes from healthy mice. This was felt to be very important because immune response against tumours initially occurs in sentinel nodes. We showed that the number of total cells was slightly increased in the SNs of tumour bearing mice, as compared to the healthy controls. There were no significant differences in the numbers of CD4+ or CD8+ cells in SNs before and after tumour inoculation. However, the number of B cells significantly increased after tumour injection, which explains the slight increase in the number of total SN cells. Most recently, it has been reported that IL-17 from CD4+ cells plays an important role in B-cell development (47, 56). The B cells' increase may be a consequence of Th determination. Th1 cells activate a cellular immunological response through increased IFN–γ and IL-2 production (57, 58), while Th2 cells suppress cellular immunological responses and promote mainly humoral immunity through increased IL-4, IL-5 IL-10 and IL-13 production (59, 58, 60).

In addition, we also investigated, in vitro, the cytotoxicity of sentinel lymph node cells. The cytotoxic capacity of SN cells was tested 13 days after tumour inoculation. We have shown higher cytotoxic activity in cells from tumour bearing mice as compared to untreated mice (57,13 vs. 39,83%). We believe that the difference in tumour-induced and spontaneous cytotoxicity is due to adaptive immunity. Th1-polarised cells secrete IFN-γ, TNF-α and IL-2 (61), which enhance the cytotoxic function of CD8+ cells (57) and macrophages (58). In general, we found no differences in the number of CD4+ and CD8+ cells in SNs after tumour inoculation, but the cytotoxic capacity of the aforementioned cells was significantly increased.

Regulatory T cells (Treg) are important in the control of the immune response (62). The majority of Treg lymphocytes express high levels of interleukin-2 (IL-2) receptor α chain (CD25) and transcription factor FoxP3 (critical for the development and function). Further, they constitute 2-3% of CD4+ human blood T cells (63). Treg lymphocytes express CTLA-4 and membrane bound TGF-β, which inhibit cytokine production and the responses of effector lymphocytes (35). They also secrete immunosuppressive cytokines such as IL-10 and TGF-β. Treg cells are a key contributor to the maintenance of immune tolerance and regulate immune responses in autoimmune diseases, graft-versus-host diseases, allograft rejections and allergies (64).

In addition, Tregs have an important role in cancer development. Cancer cells can modulate host anti-tumour immune responses indirectly, through the activation of Treg lymphocytes. Tumours promote the accumulation of immunosuppressive Treg lymphocytes in the tumour bed or in the blood. Patients with breast (65), liver (66), gastric and esophageal cancer (67) have higher numbers of Tregs in peripheral blood as compared to healthy controls. Furthermore, increased numbers of tumour-infiltrating Tregs have been demonstrated in hepatocellular (66), lung (68), ovarian (69), gastric, esophageal (67), and, more recently breast cancer (70).

Recent studies showed that Tregs play an important role in tumour growth by suppressing anti-tumour T-cell immunity (69, 71). The accumulation of Tregs within the tumour microenvironment effectively prevents tumour destruction (72) via the inhibition of CD8+ T cell function (73). The loss of regulatory function from the depletion of tumour-induced Treg lymphocytes may enhance the effector anti-tumour response, thereby resulting in tumour rejection (73-75). In our study, we showed that CD4+Foxp3+ cells numbers were decreased during tumour progression, which is thought to facilitate anti-tumour immunity.

We provide evidence suggesting that tumour progression may enhance the anti-tumour response in a model of primary breast tumours as well as pulmonary and liver metastases. This was reflected through the production of proinflammatory cytokines, the cellular composition of draining lymph node cells and cytotoxic activity. It remains to be formally shown whether Th-17 cells play a protective role in anti-tumour immunity in mammary carcinoma.

ACKNOWLEDGMENTS

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THE EFFECT OF HOMOCYSTEINE THIOLACTONE ON ACETYLCHOLINESTERASE ACTIVITY IN RAT BRAIN, BLOOD AND HEART

Milan Petrovic[1], Ivan Fufanovic[2], Iris Elezovic[2], Mirjana Colovic[3], Danijela Krstic[4], Vladimir Jakovljevic[5], Dragan Djuric[6]
[1]Clinic of Abdominal and Endocrine Surgery, Military Medical Academy, Belgrade, [2]School of Medicine University of Belgrade,
[3]Institute for Nuclear Sciences "Vinca", [4]Institute for Chemistry in Medicine, School of Medicine University of Belgrade,
[5]Department of Physiology, Faculty of Medicine University of Kragujevac,
[6]Institute of Medical Physiology „Richard Burian", School of Medicine University of Belgrade

EFEKTI HOMOCISTEIN TIOLAKTONA NA AKTIVNOST ACETILHOLINESTERAZE U MOZGU, KRVI I SRCU PACOVA

Milan Petrović[1], Ivan Fufanović[2], Iris Elezović2, Mirjana Čolović[3], Danijela Krstić[4], Vladimir Jakovljević[5], Dragan Đurić[6]
[1]Klinika za abdominalnu i endokrinu hirurgiju, Vojnomedicinska akademija, Beograd, [2]Medicinski fakultet Univerziteta u Beogradu,
[3]Institut za nuklearne nauke "Vinca", [4]Institut za hemijsku medicinu, Medicinski fakultet Univerziteta u Beogradu,
[5]Katedra za fiziologiju, Medicinski fakultet Univerziteta u Kragujevcu ,
[6]Institut za medicinsku fiziologiju „Richard Burian", Medicinski fakultet Univerziteta u Beogradu

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ABSTRACT

Limited data exist in the literature regarding the effects of homocysteine thiolactone on the activity of the acetylcholinesterase (AChE) in the blood, and practically no data exist regarding the influence of homocysteine thiolactone on the enzyme in the brain and heart. Taking into consideration the importance of hyperhomocysteinemia in clinical practice, it has been thought to be of particular interest to examine the effect of homocysteine thiolactone on the activity of AChE in the rat's blood, brain and heart. In this study, male Wistar rats (weighing 250-300g) were used, and they were divided into two groups; one served as a control group and receieved a placebo (1 ml 0.9 % NaCl, i.p.), while the other group received a homocysteine thiolactone solution (5.5 mmol/kg b.m., i.p.). An hour after the administration, the rats were euthanized by decapitation, heir tissues were harvested, buffered, and homogenized in a phosphate buffer (pH 8). The concentration in the tissue homogenates was 20 mg of tissue per 1 ml of buffer. The buffered and homogenized parts of the tissues were used as substrates for spectrophotometric measurements. The AChE activity was then measured by the Ellman method. Statistical analysis was conducted using a one-way ANOVA test, and the intergroup comparisons were performed using a Bonfferoni test. The results showed a significant reduction in AChE activity in all tissues obtained from the animals treated with homocysteine thiolactone compared to the enzyme activity of the control group. In addition, the results also showed that the blood enzyme activity inhibition was the lowest (12%), while the enzyme activity was slightly higher in the brain (27.8%) and heart specimens (86.3%). It was concluded that homocysteine thiolactone significantly inhibited AChE activity in the heart and brain tissue, but not in the blood of the rat.

Keywords: *acetylcholinesterase, homocysteine thiolactone, specific enzyme activity*

SAŽETAK

U literaturi je nađeno malo podataka o uticaju homocisteina na aktivnost acetilholinesteraze u krvi, a direktnih nalaza o uticaju homocistein tiolaktona u mozgu i srcu nema. S obzirom na medicinski značaj pojave hiperhomocisteinemije, smatrali smo da je od interesa da se ispita uticaj D,L-homocistein tiolaktona na aktivnost acetilholinesteraze u krvi, mozgu i srcu pacova. U eksperimentu su korišćeni pacovi mužjaci soja Wistar (telesne mase 250-300g) podeljeni u dve grupe: jedna grupa je bila kontrolna i dobijala placebo (1ml 0,9 % NaCl, i.p.), dok je druga grupa dobijala rastvoreni homocistein tiolakton (5,5mmol/kg t.m, i.p.). Sat vremena po aplikaciji pacovi su dekapitovani, dobijena tkiva su zatim puferovana i homogenizovana u fosfatnom puferu (pH 8). Koncentracija tkiva u homogenatu iznosila je 20mg tkiva po ml pufera. Puferovani i homogenizovani delovi tkiva su korišćeni kao supstrat za spektrofotometrijska merenja. Zatim se pristupilo merenju aktivnosti acetilholinesteraze, koja je merena metodom po Ellmanu. Statistička obrada podataka urađena je jednofaktorskom analizom varijanse, a međugrupna poređenja Bonferonijevim testom. Rezultati pokazuju da postoji značajno smanjenje aktivnosti enzima acetilholinesteraze u svim tkivima uzetih od pacova tretiranih homocistein tiolaktonom, za razliku od aktivnosti enzima kontrolne grupe koja je dobijala placebo, i to: u krvi je utvrđena najmanja inhibicija specifične aktivnosti (12%), u mozgu nešto veća (27,8%), dok je u srcu najveća (86,3%). Na osnovu dobijenih rezultata zaključeno je da homocistein tiolakton u značajnom procentu inhibira aktivnost acetilholinesteraze u mozgu i srcu, ali ne i u krvi pacova.

Ključne reči: *acetilholinesteraza, homocistein tiolakton, specifična enzimska aktivnost*

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Correspondence to:
Prof. Dr. Dragan Đurić / Institute of Medical Physiology "Richard Burian" / School of Medicine University of Belgrade / Višegradska 26/II, 11000 Belgrade
Tel. +381 11 36 07 074 / Fax. +381 11 36 11 261 / drdjuric@eunet.rs

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme that rapidly hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses, including the neuromuscular junction. Recent surveys have highlighted the enormous importance of AChE in processes such as the growth of cholinergic and non-cholinergic neurons, as well as in processes related to extraneural tissues, including the inhibition of hematopoietic stem cell differentiation, connection of amyloid fibres, the influence on apoptosis, and neoplasma growth (1,2). AChE is transcribed from only one gene, but due to a variety of post-translational processes, it exists in a variety of isoforms and is also present in numerous tissues. By combining a number of different isoforms, it is possible to acquire more complex structures, mainly in the form of dimers and tetramers. These forms are connected to the plasma membrane with an "anchor" of a glycophosphatidylinositol structure, while the form on a neuromuscular connection is represented by appropriate anchor proteins (sequence WAT, consisting of aromatic amino acids on the enzyme) to a collagen-like domain (PRAD, CoQ protein) (3,4). Homocysteine thiolactone is the cyclic metabolite of homocysteine, generated in an organism under oxidative stress conditions and the lack of vitamin B12 and/or folic acid. The most common route of its creation comes from the metabolism of folic acid accompanied by vitamin B12 where homocysteine is created from methionine. Another route for generating homocysteine is by methylation with betaine homocysteine-methyltransferase. Homocysteine thiolactone is a very reactive metabolite that has been viewed to be enormously important in the pathogenesis of cardiovascular diseases, diabetes, and osteoporosis, as well as in various diseases of the central nervous system including Alzheimer, neural tubus defects and schizophrenia (5). The mechanisms of the effect of homocysteine thiolactone on the above mentioned disorders are not known, but it is possible that homocysteine acts, as a reducing agent, reacts with the sulfhydryl groups of certain molecules, thereby changing their structure, adhesion and signalling within cells. Another mechanism could be an increase in the quantities of S-adenosyl methionine resulting in a reduction of gene methylation, and consequently a reduction in gene expression (6).

Little is known about the influence of homocysteine thiolactone on AChE activity in the blood, while descriptions of the effect of homocysteine thiolactone on the brain and heart are practically non-existent. With respect to the clinical importance of hyperhomocysteineamia, it is crucial to investigate the influence of D,L-homocysteine thiolactone on the activities of the AChE enzyme in the blood, brain and heart of a rat.

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 250-300g were used in the experiment and were studied at 10 weeks of age. The animals were kept in a standard laboratory environment at a temperature of 22oC. Water and food were provided ad libitum.

Tissue preparation

The animals were divided into two experimental groups, six rats per group. The first group was the control group, and the second was treated with homocysteine thiolactone (Sigma Chemical Co. USA). The animals were cared for in accordance with the codes for laboratory animals established by the School of Medicine, University of Belgrade, and in compliance with the Committee of Ethics related to the work with experimental animals. Homocysteine thiolactone was dissolved in buffered 0.9% NaCl at pH 7.4. A solution (5.5 mmol/kg of body weight) of homocysteine thiolactone (1 ml) was administered intraperitoneally. The control group was given a placebo intraperitoneally (1 ml 0.9% NaCl).

Sixty minutes later, the rats were euthanized by decapitation. Whole brains and hearts isolated from the rats were rinsed in a phosphate buffer pH 8.0, and the blood was stored in test tubes coated in heparin. The brains and the hearts were homogenized in cold phosphate buffer (pH 8.0). The final tissue concentration was 20 mg tissue per ml buffer.

Biochemical determination

AChE activity was determined by Ellman's method (7). The incubation mixture contained: 20µl brain homogenate in 600µl of the phosphate buffer pH 8.0; 40µl heart homogenate in 580µl of the phosphate buffer pH 8.0; 50µl heparinized blood (diluted in sodium chloride 1:100) in 570µl of the phosphate buffer pH 8.0. The mixture was incubated at 37 oC for 10 minutes. A volume of 20µl 5,5'-dithionitrobenzoic acid (DTNB) (Sigma Chemical Co, USA) and 10µl of acetylcholine iodide (Sigma Chemical Co, USA), used as substrates, was added, and the reaction was started. The reaction was monitored spectrophotometrically (Gilford Instrument, Model 250) by an increase in the absorbance (ΔA) at 412nm. An assay, without the tissue homogenate, was used as a blank probe. The measurements were assessed with double probes, and the specific AChE activity was calculated as ΔA (min x mg tissue) for the brain and heart, and ΔA (min x µLblood) for blood.

Statistical analyses

Values are presented as means ± SD. Statistical analyses were performed using a monofactorial analysis of variance, as well as Bonferroni test. P values less then 0.05 were considered to be significant.

Chemicals used

All chemicals were of p.a. grade quality. D,L-homocysteine thiolactone, acetylcholine-iodide (ASChI) and 5,5-dithiobis(2 nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (USA).

RESULTS

The AchE activity determined in homogenized whole brain, heart and blood from non-treated rats (control group) are presented in Table 1. The AchE activities are significantly different between the different types of rat tissues. Higher enzyme activities were recorded in the brain

Acetylcholinesterase activity (means ± SD)

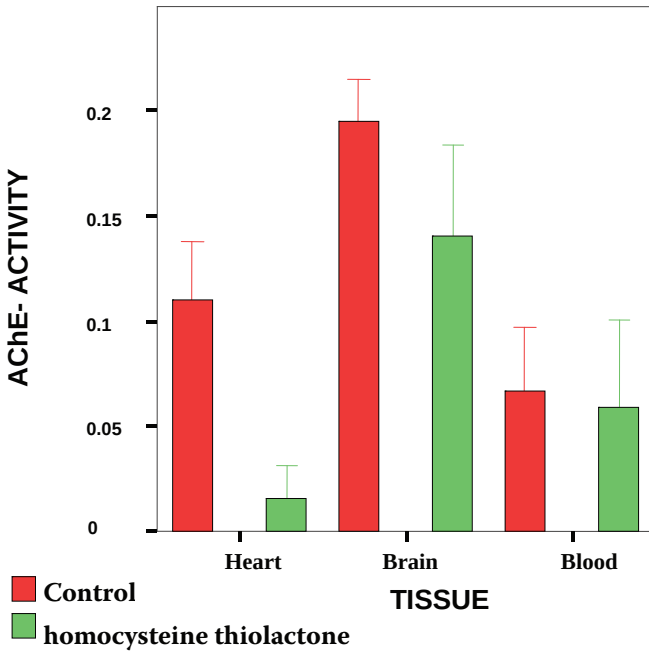
n=6		p-value[a]	p-value[b]		p-value[a]	p-value[b]
Tissue	Control			Treated		
Heart	0.110±0.028	< 0.001	< 0.05 vs. brain and blood	0.015±0.016**	<0.001 vs. control	<0.001 vs. control
Brain	0.194±0.020	< 0.001	< 0.05 vs. brain and blood	0.140±0.044*	<0.01 vs. control	NS
Blood	0.067±0.030	< 0.001	< 0.05 brain and heart	0.059±0.041	NS	NS

a- statistical test applied : ANOVA
b- statistical test applied : Bonferroni test

(0.194±0.020) and in the heart (0.110±0.028). The lowest activity was recorded in the blood (0.067±0.030). All enzyme activities from homogenized tissues of rats treated with homocysteine thiolactone were decreased compared to the control group. Moreover, these activities were significantly lower compared to control values for the heart (by 86.3%) and the brain (by 27.8%) (Table 1, Fig 1). However, there was no evidence of a significant difference in the AchE activity for blood in the treated group (0.059±0.041) compared to control values (0.067±0.030) (Table 1, Fig 1).

DISCUSSION

Homocysteine thiolactone inhibits the activity of AChE, and specific AChE activities are different in different types of tissues (brain, heart and blood) in the rat (8). A similar ration of the specific AChE activities in the experimental animals has been observed (9). In this paper, we described the influence of homocysteine thiolactone on AChE activities in the brain, heart and blood of the rat. Our results clearly show that an acute treatment with homocysteine thiolactone causes a decrease in the AChE activity, while the sensitivity of AChE in the brain, heart and blood in the presence of the tested substance mutually differ.



The highest sensitivity of AChE, i.e., the highest inhibitory effect of homocysteine thiolactone on the enzyme activity, was obtained in the heart, where the activity of this enzyme was decreased by 86.3% compared to the control value. This treatment resulted in the inhibition of AChE in the brain by 27.8% compared to the control group, while the lowest sensitivity was obtained for AChE in the blood. The observed decrease in the specific activity of AChE in the blood was 12% compared to the control group, and cannot be considered a statistically significant change compared to the control group. Homocysteine thiolactone in vitro inhibits the activity of butyrylcholinesterase (containing a similar structure to AChE) in the blood of the rat; this inhibition is proportional to the concentration of the inhibitor (10). Furthermore, according to the kinetic analysis of inhibition, homocysteine thiolactone and the substrate bind to the same site in the enzyme (10).

Another group of researchers believe that homocysteine thiolactone produces tight thioester connections (8). A significant decrease of AChE activity corroborates our results. In addition to the stated (un) competitive inhibition of AChE with homocysteine thiolactone, the obtained inhibition of the tested enzyme could be explained by the fact that the presence of the increased concentration of homocysteine thiolactone produces an increase in the production of oxidative free radicals, i.e., the obtained decrease of the enzyme activity tends to be a consequence of the oxidative stress in the functional groups on the enzyme (8, 11). Providing that hyperhomocysteinemia (as well as the increased concentration of homocysteine in tissues) is present in a variety of pathological disorders (5,11). In addition to the fact that the mechanistic role of this metabolite in the initiation and development of disease processes is not known, the results obtained here suggest that the inhibition of AChE with homocysteine could be one of the most possible mechanisms responsible for the pathogenesis of the diseases stated herein.

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THE ROLE OF PHD TEACHERS IN MEDICAL EDUCATION

Dhastagir Sultan Sheriff[1] and S. Omer Sheriff [2]
[1]Department of Biochemistry, Al Arab Medical University, Benghazi, Libya and
[2]Priyadarshini Dental College, Chennai, India

"Most ideas about teaching are not new,but not everyone knows the old ideas."
Euclid, circa 300BC

Many understand the value of doctorates in the field of education, whether it be science, engineering or medicine. The field of research and its development depend upon the doctorates who devise, guide and conduct research projects and also teach. Therefore, it is worthwhile to understand the status of doctorates in education, particularly in medical education. With this view in mind, an attempt has been made to outline the role of PhD teachers in medical education.

What does the degree Doctor of Philosophy (PhD) mean?

The term *doctorate*, derived from the Latin word *docere* meaning "to teach", is shortened from the full Latin title *licentia docendi* meaning "teaching license".

A doctorate is an academic or professional degree that, in most countries, represents the highest level of formal study or research in a given field. In some countries it also refers to a class of degrees that qualifies the holder to practice in a specific profession (such as law or medicine). The best-known example of the former is the PhD (Doctor of Philosophy), while examples of the latter include the U.S. degree Doctor of Medicine, a doctoral degree for physicians.

The Doctor of Philosophy, abbreviated PhD, or alternatively D Phil, from the Latin meaning "teacher of philosophy", is an advanced academic degree awarded by universities.

Historically, it was thought that PhD teachers or scientists had been trained to become university professors. The United States is reported to be the greatest producer of PhDs, generating about twice as many as the next leading country, Germany. Countries with small populations, such as Canada and Switzerland, produce relatively few PhDs. However, many other countries that have huge populations, such as India and China, also produce relatively small numbers.[1]

Types of PhD:

PhD students or doctorates usually fall in one of several categories. Research doctorates conduct academic research in a stipulated period of time and submit a thesis with a substantial body of original research. At a higher level in India, a Doctor of Science (DSc) or Doctor of Letters (Dlitt/LittD) is awarded by a university based on the submission of a portfolio of published research of a very high standard. Some universities award honorary doctorates to an individual for his/her contributions to a particular field or for philanthropic efforts. In certain European countries, habilitation is the highest academic qualification a person can achieve by his/her own pursuit and is still used for academic recruitment purposes in many countries within the EU. It involves either a new, long thesis (a second book) or a portfolio of research publications. Habilitation demonstrates independent and thorough research, experience in teaching and lecturing and, more recently, the ability to generate funding within the area of research. The habilitation is regarded as a senior post-doctoral qualification completed many years after the research doctorate and can be necessary for a Privatdozent or for a professor position in Germany.[2] A similar system traditionally exists in Russia. Previously in the Russian Empire the academic degree doctor of science (doktor nauk) marked the highest academic degree that can be achieved by an examination. This system was generally adopted by the USSR/Russia and many post-Soviet countries.

Research doctorates are awarded in recognition of academic research that is (at least in principle) publishable in a peer-reviewed academic journal. Criteria for award of research doctorates vary somewhat throughout the world but typically require the submission of a substantial body of original research undertaken by the candidate. This may take the form of a single thesis or dissertation or possibly a portfolio of shorter project reports and is usually assessed by a small committee of examiners appointed by the university and often an oral examination of some kind. In some countries (such as the US) there may also be a formally taught component, which typically consists of graduate-level courses in the subject in question as well as training in research methodology.

The minimum time required to complete a research doctorate varies by country and may be as short as three years (excluding undergraduate study), although it is not uncommon for a candidate to take up to ten years to complete his/her degree.

In Spain, Doctor Degrees are regulated by Royal Decree (R.D. 778/1998) (Real Decreto in Spanish). They are granted by the university on behalf of the King, and the diploma has the force of a public document. The Minis-

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Correspondence to: dhastagir@yahoo.ca

try of Science keeps a National Registry of Theses called TESEO. According to the National Institute of Statistics (INE), less than 5% of M.Sc. degree holders are admitted to PhD programs, and less than 10% of 1st year PhD students are finally granted a Doctor title. All doctoral programs are research in nature. A minimum of five years of study are required, divided into two stages:

1) A 3-year long period of studies, which concludes with a public dissertation presented to a panel of three professors. If the project receives approval from the university, he/she will receive a "Diploma de Estudios Avanzados" (partially qualified doctor).

2) A 2-year (or longer) period of research. Extensions may be requested for up to ten years. The student must write his thesis presenting a new discovery or original contribution to science. If approved by his "thesis director", the study will be presented to a panel of five distinguished scholars. If approved, he will receive the doctorate. A Doctor Degree is required to apply to a teaching position at a university.

The combined MD-PhD program in the USA and, more recently, in Australia aims to produce clinician–scientists committed to pursuing research that reflects their experience of clinical practice.[3]

In Libya the faculty are required to have a PhD awarded by a professional university in the subjects they teach. For example, a PhD awarded by a medical university is required for teaching medical students and other respective universities or faculties for other subjects.

Role of a PhD in medical education:

Medical education is the science behind the teaching and learning in medicine. It has developed from problem identifier domain to that of solution provider. Abraham Flexner of the Carnegie Foundation placed emphasis on the scientific basis of medical practice. Therefore, formal analytic training, integral to the natural sciences, was thought to be inculcated in the intellectual training of physicians.[4] Clinical phase education, it was thought, would encourage physicians to consider pursing research to provide better care for their patient.

Transformation from patient care to molecular research

Currently, there is a real transformation of thinking about teaching, which is considered subordinate to research. This transformation is due to the development of the "publish or perish" attitude. Previously, the integration of investigation with teaching and patient care made the field of medicine dynamic. However, the shift from patient care to molecular events has diminished the standard of clinical teaching in favour of laboratory research.[5] This shift is a result of economic need wherein to generate revenue, the physician is forced to provide care for paying patients. Therefore, clinical teachers have less time available for teaching. The importance of responsible clinical teaching is vital for medical students to become accomplished, responsible and service minded. To accomplish this goal, medical education needs to balance knowledge, skills and values during the students' period of learning. Instead, theoretical, scientific knowledge of formal learning has superseded clinical, patient-oriented education. Now, the knowledge of medical research is grounded more in basic medical sciences.[6] Therefore, there is currently a cry for more clinical teachers to teach students in a clinically oriented, patient-centred fashion.

Therefore, to give importance to teaching and to provide time for clinicians to be more patient-centred, PhD teachers are trained to teach basic medical sciences.

These PhD scientists and teachers are considered to be the torchbearers of not only teaching these subjects but also developing basic biological science research programs in medical schools. In this context, the PhDs of the medical faculty with knowledge of clinical subjects are considered scientist cum teachers.[7,8]

A medical educator is usually a medical scientist and clinician with a special interest and expertise in medical education. A medical educator may be someone who is:

a) Specially skilled in teaching
b) A person trained in the educational theory and practice in the context of medicine
c) An administrator in medical institution

Therefore, the role of PhD teachers in the faculty of medical schools is clear-cut. They teach and develop research programs in medical institutes. In a changing social context of medicine, basic science research and its teaching need to develop because they form the foundation stone of basic and applied research in the field of medicine.

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METHODS FOR DERIVATION OF HUMAN EMBRYONIC STEM CELLS

Biljana Ljujic[1] and Miodrag Stojkovic[1, 2]
[1]Centre for Molecular Medicine, Faculty of Medicine, University of Kragujevac, Serbia;
[2]Centro de Investigacion Principe Felipe, Valencia, Spain

METODI ZA DOBIJANJE HUMANIH EMBRIONALNIH STEM ĆELIJA

Biljana Ljujić[1] i Miodrag Stojković[1, 2]
[1]Centar za molekulsku medicinu, Medicinski fakultet, Univerziteta u Kragujevcu, Srbija;
[2]Istraživački centar Princ Filip, Valensija, Španija.

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ABSTRACT

Human embryonic stem (hES) cells are self-renewing cells that can give rise to many types of cells found in the body. These cells can only be extracted from pre-implantation embryos. The timing of derivation of hES cells from the preimplantation embryo, as well as the means by which hES cells are isolated, differs among lines. Therefore, the stage of development from which hES cells are derived may be crucial for their use as models of differentiation and in regenerative medicine. In this review, we summarise methods by which hES cells are derived from embryos at different stages. In addition, we review methods for generating pluripotent cells from adult cells and evaluate the potential utility of ES-like cells or reprogrammed induced pluripotent cells (iPSC).

Keywords: *derivation, human, embryonic, stem cells*

SAŽETAK

Humane embrionalne stem ćelije (hES) mogu se dobiti samo iz pre-implantacionih embriona. To su ćelije koje imaju mogućnost samoobnavljanja iz kojih kasnije nastaju razne vrste ćelija u telu. Postoje razlike u vremenskom periodu kada se hES dobijaju iz pre-implantiranog embriona i u načinu kako se hES ćelije izoluju. Faza razvoja iz koje potiču hES ćelije može biti ključna u njihovoj upotrebi kao modela diferencijacije i u regenerativnoj medicini. U ovom pregledu predstavićemo sve metode derivacije humanih embrionalnih stem ćelija iz embriona u različitim fazama i metode derivacije pluripotentnih ćelija iz adultnih ćelijskih izvora. U nastavku, ocenićemo ulogu stem ćelija sličnih embrionalnim ili reprogramiranih ćelija sa ćelijski indukovanom pluripotentnošću (iPSC).

Ključne reči: *izolovanje, humane, embrionalne, matične ćelije*

Abbreviations:

ES cells: embryonic stem cells;
hES cells: human embryonic stem cells;
iPSC: induced pluripotent cells;
ICM: inner cell masses;
MEF: murine embryonic fibroblasts;
pES: parthenogenetic human embryonic stem cell;
SCNT: somatic cell nuclear transfer;
SSEA-1, SSEA-3 and SSEA-4:
stage-specific embryonic antigens 1, 3 and 4;
TRA-1–60 and TRA-1–81:
tumour-resistance antigens-1-60 and 1-81.

Skraćenice:

ES: embrionalne matične ćelije;
hES: humane embrionalne matične ćelije;
iPSC: indukovane pluripotentne matične ćelije;
ICM: unutrašnja ćelijska masa;
MEF: mišji embrionalni fibroblasti;
pES: partenogenetske humane matične ćelije; ;
SCNT: somatski nuklearni transfer;
SSEA-1, SSEA-3 i SSEA-4:
stadijum specifični embrionalni antigeni 1, 3 i 4;
TRA-1–60 i TRA-1–81:
tumor-rezistentni antigeni.

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Correspondence: Biljana T. Ljujic, MD / Centre for Molecular Medicine,
Faculty of Medicine, University of Kragujevac,Svetozara Markovica 69, 34 000 Kragujevac, Serbia,
Phone: +38134306800; / Fax: +38134306800 ext 112; / E-mail: bljujic74@gmail.com

INTRODUCTION

Human ES (hES) cells are progenitor cells with the capacity to divide and give rise to identical stem cells (symmetrical division) or to specialise and form specific cells of the somatic tissues (asymmetrical division) (1). Human ES cells can only be derived from preimplantation embryos and have a proven ability to differentiate into all cell types of the adult organism (termed "pluripotent"). Human ES cells have been derived from morulae (2), single blastomeres (3), embryos at the blastocyst stage (4, 5), and parthenogenetic embryos (6). There are differences in the timing of derivation of hES cells from the preimplantation embryo as well as the means by which the hES cells are isolated (Figure 1). Despite these differences in the derivation process, all hES cell lines that have been derived to date exhibit similar expression patterns of standard stem cell markers.

Markers that are now recognised as important for determining the pluripotent potential of hES cells include Oct4, Nanog, Sox2, Foxd3, Rex1, and UTF1 transcription factors; TERF1, CHK2, and DNMT3 DNA modifiers; the surface marker, GFA1; the growth factor, GDF3; the TDGF1 receptor; as well as Stella and FLJ10713 (7). To characterise hES cells, it is common to report one or more of the following: Oct4 expression; alkaline phosphatase and telomerase activities; the presence of stage-specific embryonic antigens 3 and 4; the expression of hESC antigens TRA-1–60, TRA-1–81, GCTM-2, TG-30, and TG-343; and/or the expression of CD9, Thy1, and the class 1 major histocompatibility complex (MHC-1) (8).

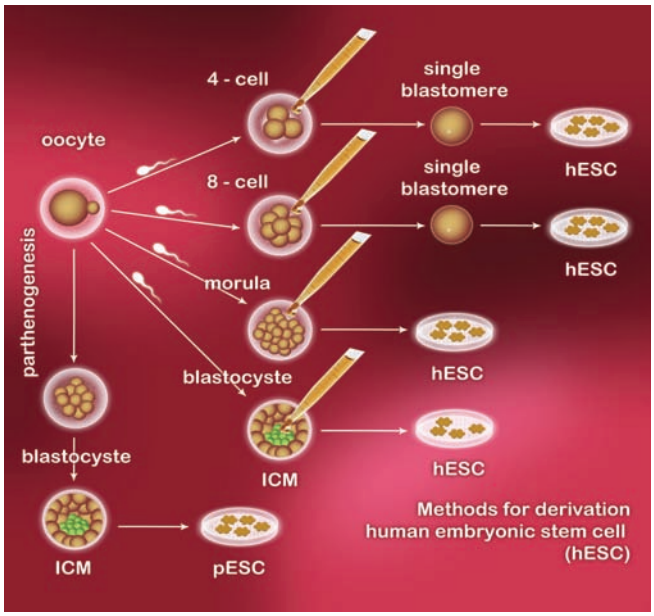

Figure 1. Methods for the derivation of human embryonic stem cells (hESC) from different stage embryos and method for derivation parthenogenetic human ES cell lines (pES). These are four embryo stages showed in the literature to give rise to hESC lines. Fertilized oocytes cultured in vitro give rise to morula (4–16 cells) and later to blastocyst formation (16–40-cell stage). Human ESC lines can be derived from single blastomere, the whole morule or complete ICM. Parthenogenesis is a form of asexual reproduction in which eggs can develop into embryos without being fertilized by sperm. Isolated ICM from parthenogenetic embryos give rise to pES lines that displayed the typical ES cell morphology. ICM: inner cell masses.

Stem cells can either continue to grow in a pattern of prolonged self-renewal or differentiate. This choice of fate is highly regulated by both intrinsic signals and the external microenvironment (9, 10). For example, hES cells can be induced to differentiate into functional cardiomyocytes (11), pancreatic islet cells (12), dopamine-producing neurons (13), hepatic cells (14) and hematopoietic cells (15).

As part of the historical introduction of the term "ES cell", Thomson et al. (1998) (16) proposed that the essential characteristics of ES cells should include (I) derivation from the preimplantation or preimplantation embryo, (II) prolonged undifferentiated proliferation, and (III) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. Human ES cells are also immortal, expressing high levels of telomerase, the protein that maintains the telomeres during each cell division and, as a result, prevents cells from undergoing senescence. Almost all human ES cells that have been isolated to date have normal karyotypes; however, chromosomal changes could occur during derivation, culturing, and routine karyotyping of cells. In 2004, Draper et al. (17) reported amplifications of chromosomes 17q and 12p in two hES cell lines that were cultured for periods of several months (22–60 passages).

Following their isolation, hES cells are usually cultivated on inactivated mouse fibroblasts. To avoid contamination from animal cell components, cells of human origin can be used as feeder layers (18). The use of human feeder cells, such as foetal muscle, foetal skin, adult fallopian tube epithelial cells (19-21), foreskin fibroblasts (22), adult marrow cells (23), adult endometrial cells (24) and hES cell-derived fibroblasts (25) has proven useful for generating viable hES cells (26). Yet, while these systems eliminate direct contact between hES cells and feeder cells, they are limited by low success rates in the initial transfer of hES cells from feeder to feeder-free conditions (26) and can lead to the development of mixed populations of undifferentiated and differentiated hES cells (27-29). More recently, it has been demonstrated that hES cells can sustain their undifferentiated states for over six months in the absence of feeder cells, which represents a potentially crucial development (30).

Another potential source of pluripotent stem cells is from induced somatic cell differentiation (31). IPSCs are adult somatic cells that have been genetically modified and reprogrammed to undergo a process of dedifferentiation.

In this review, we summarise methods for the derivation of hES cells from embryos of different stages. We also review methods for deriving pluripotent cells from various sources of adult cells. The recent demonstration that pluripotent cells can be derived from different embryonic and adult cell sources will provide unique opportunities to comparatively examine the key factors and conditions that facilitate the expansion of pluripotent cells.

Derivation of human embryonic stem cells

In 1998, Thomson et al. (16) reported the first successful creation of hES cells. Two years later, methods were further developed and refined by Reubinoff et al. (32) to successfully establish hES cell lines. These methods were similar to those described by Thomson et al. in 1998, which involved the isolation of inner cell masses (ICM) from human blastocysts by immunosurgery and co-culture with mitotically inactivated murine embryonic fibroblasts (MEFs). So far, more than 500 successful hES cell lines have been isolated by various groups worldwide using human fresh or frozen morulae and/or embryos in the blastocyst stage. Since then, rapid progress has been made, and numerous studies have described the derivation of new hES cell lines, including methods for their derivation as well as methods for growing both undifferentiated hES cells and their differentiated progeny.

The efficiency of deriving hES-cell lines varies vastly between different laboratories, depending on the isolation conditions, the experience of the group performing the procedures and the quality of the embryos used (8); however, a comparison of success rates between groups is quite difficult because of the differences in how groups report their data. For example, success rates can be reported per cleavage-stage embryo or per blastocyst used, per total number of embryos entered into a given program, or per successfully plated blastocyst or inner cell mass.

Derivation of human embryonic stem cells from morula stage embryos

Human ES cells are derived from the inner cell mass of the blastocyst, although the embryos in other developmental stages have also been used as sources of ES cells. Studies in the mouse have shown that it is possible to isolate ES cells during a limited time period in early development. Mouse ES cell lines have been obtained from the eight-cell blastocyst stage to the compacted morula stage (33-35). Human ES cells have also been obtained recently from human morulae by initially culturing the morulae underneath a feeder layer (2). Similar to ES cells originating from blastocysts, the morula-derived ES cells express the same ES cell-specific markers, including Oct-4, TRA-2-39, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 and were shown to spontaneously differentiate into a variety of cell types in vitro.

Derivation of hES cells from single blastomeres

It is known that if one or two cells are missing from a preimplantation embryo, the remaining cells can compensate to form a whole embryo. A single cell can be isolated from the cleavage stage embryo and used to create a cell line while the rest of the embryo can be transferred back to the uterus to give rise to a foetus (36). By growing the single blastomere overnight, the resulting cells can be used for both genetic testing and stem cell derivation without affecting the clinical outcome of the procedure (3).

Human ES cell lines derived from 8-cell stage human blastomeres have been reported (3, 37), but Geens et al. (2009) recently reported the derivation of two hES cell lines from 4-cell stage human blastomeres (38). These were top quality 4-cell stage embryos with equal-sized blastomeres and less than 10% fragmentation. The single blastomere without the zona pelucida was cultured in solitude. Three or four days after fertilisation, the blastomere gave rise to blastomere-derived embryos. The embryos were washed and plated in individual dishes coated with mitomycin C-inactivated MEFs and cultured in hES cell medium. Whenever an outgrowth was present, it was mechanically passed and cultured in the same manner as with blastocyst-derived hES cells. Previous studies have addressed the possibility of obtaining hES cells from early cleavage embryos, i.e., with less than ten cells (3, 37, 38); however, recently, Feki et al. (2008) reported the derivation of an hES cell line from a single blastomere of an arrested four-cell-stage embryo (39). This type of study may provide insight into the different success rates of deriving abnormal and normal hES cell lines, particularly in the case of mosaic embryos. As a result, this work could improve scientific knowledge of genetic anomalies and their possible involvement in causing embryo arrest.

Derivation of human embryonic stem cells from blastocyst stage embryos

Human blastocyst-derived, pluripotent cell lines are described to have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that distinguish primate embryonic stem cells from other early lineages (16). This method uses embryos generated for in vitro fertilisation (IVF) that are no longer needed for reproductive purposes. Day-5 blastocysts are used to derive ES cell cultures. A normal day-5 human embryo in vitro consists of 200 to 250 cells. Most of the cells comprise the trophectoderm. For deriving ES cell cultures, the trophectoderm is removed, either by microsurgery or immunosurgery (in which antibodies against the trophectoderm help break it down, thus freeing the inner cell mass). At this stage, the ICM is composed of only 30 to 34 cells (40). Inner cell masses are pluripotent and can give rise to many types of somatic cells. Because hES cells can be derived from the ICMs of expanded blastocysts, not only blastocyst quality but also optimal ICM size and shape are crucial to this process. Therefore, methods for the derivation of hES cells depend on the morphology of the blastocysts and the appearance of their ICMs (41). When blastocysts possess large and distinct ICMs, the most frequently used method is immunosurgery (42-45). In this method, blastocysts are incubated in the presence of anti-human serum and subjected to antibody complement treatment to lyse the trophectoderms. The lysed trophectoderms are then repeatedly passed through a pipette to separate the trophectoderms from the ICMs and the isolated ICMs are cultured on inactivated feeder cells. A potential shortcoming to immunosurgery is the exposure of the blastocysts

to animal antibodies, which can negatively impact future developments in clinical settings (46). In cases in which expanded blastocysts develop but exhibit smaller ICMs, partial-embryo cultures were used (41). These blastocysts were treated with pronase to dissolve their zona pelucidae and ICMs were surgically isolated. Arrested embryos of poor quality can also be used for the derivation of hES cells (4, 47, 48). This method employs whole-embryo culture, in which intact developing or arrested embryos were freed from zona pellucidae and plated on inactivated mouse or human feeder cells. Several hES cell lines were derived from cloned human embryos using the whole-embryo culture method (49).

For clinical grade hES cell lines, a robust system would be required that lacks any substances of animal origin. In addition, when blastocysts contain small ICMs, mechanical isolation of ICMs can be performed to avoid loss of the ICMs (50). Several groups of researchers (50-52) developed a practical, mechanical ICM isolation procedure. Two flexible metal needles were used to open zona pellucidae and extract ICMs under a stereomicroscope. Mechanical isolation of ICMs has been reported previously (53-55) using whole blastocysts placed on feeder layers, followed by removal of the trophectoderm cells during the early stages of culture. ICMs can also be removed by laser micromanipulation techniques. Along these lines, ICMs were dissected from the trophectoderm by a laser beam and removed through the orifice of the zona pellucida (56). Moreover, laser micromanipulation was successfully applied for the isolation of ICMs and establishment of hES cell lines (57).

Derivation of human embryonic stem cells from parthenogenetic embryos

Parthenogenesis is a form of asexual reproduction in which eggs can develop into embryos without being fertilised by sperm, the so-called "virgin-birth embryos" (58). Parthenogenesis is one of the main, and most useful, methods used to derive embryonic stem cells, which may be important sources of histocompatible cells and tissues for cell therapy. In parthenotes, all genetic material originates from the maternal genome, and the resulting stem cells demonstrate only the maternal patterns of gene imprinting.

ES cell lines have been successfully established from parthenogenetically-activated oocytes in many species, including mice, cows, rabbits and nonhuman primates (59). Recently, however, the successful development of parthenogenetic human ES cell lines (pES) was also reported (6, 60-62). All of these reported lines displayed a typical ES cell morphology: a high nuclear/cytoplasmic ratio and clearly distinguished nucleoli, as well as the expression of known hES cell positive markers (SSEA-4, SSEA-3, TRA-1-60, TRA-1-80), but not the negative marker, SSEA-1. They also expressed many genes involved in maintaining a pluripotent state. These cell lines have normal karyotypes and one line was even shown to maintain a normal karyotype after more than 100 passages (6). Parthenogenetic ES cell lines also possess high levels of alkaline phosphatase and telomerase activity. Parthenogenetic activation is artificially induced by various physical and/or chemical methods (63). Parthenogenetic activation of oocytes has a few steps. First, prior to activation, granule cells around individual oocytes are removed by brief exposure to hyaluronidase. Next, electric activation is performed with an electric pulse in electrofusion medium containing mannitol, after which the oocytes are transferred into ionomycin. The activated oocytes are then cultured in cleavage medium, and pronuclear cells are observed 18 h later. Embryos are then transferred into blastocyst culture medium (6). Parthenogenetic ES cells have tremendous potential for cell therapy in clinics and may be a useful model and an experimentally verified system to study stem cell biology (including epigenetic regulation of ES cells).

Other sources of pluripotent stem cells

Stem cell researchers continue to isolate new pluripotent cells and create additional cell lines. Here we describe other sources of pluripotent stem cells, and we discuss the implications that their discovery may have on future research. One recently demonstrated method for the derivation of pluripotent stem cells is the use of induced somatic cell differentiation (31).

Derivation of human stem cells from induced somatic cell differentiation

With this method, adult somatic cells are genetically modified and reprogrammed to undergo a process of dedifferentiation by inducing the expression of pluripotency related genes (Figure 2). Human induced pluripotent stem cells, produced either by expression of OCT4, SOX2, NANOG, and LIN28 (31) or by OCT4, SOX2, c-Myc and KLF4 (64) are also remarkably similar to human ES cells. In 2008, Junying and Thomson (65) showed that OCT4, SOX2 and NANOG are sufficient to reprogram human cells. They did not predict this ability in advance, but instead carried out a very time consuming screen that eventually narrowed the list down to these three factors. These cells, designated as human iPSCs, exhibit the morphology of ES cells and express ES cell markers (66). Induced PS human stem cells have normal karyotypes, possess telomerase activity, express cell surface markers and genes that characterise human ES cells, and maintain the developmental potential to differentiate into advanced derivatives of all three primary germ layers (31). Global gene expression analyses of various iPS clones and hES cell lines identified minor differences between iPS cells and hES cells and between different iPS clones, although these differences were not greater than those found between distinct hES cell lines (31, 64). Yet, there are a few problems associated with clinical applications of these cells. The efficiency of reprogramming adult fibroblasts remains low (<0,1%), but whether this frequency reflects the need for precise timing, balance, and absolute levels of expression of the reprogramming genes, or selection for rare genetic/epigenetic changes either initially

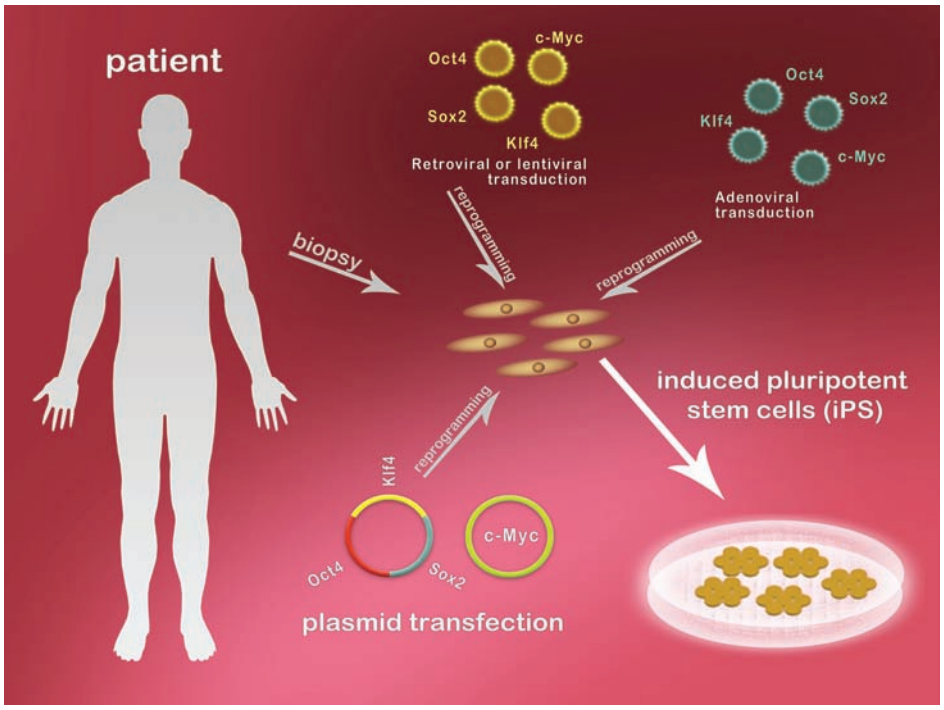

Figure 2.

Methods for derivation of pluripotent stem cell from adult somatic cells by genetic modification and reprogramming. Somatic cells are transduced with a minimal set of transcription factors required for cellular reprogramming (Oct4, Sox2, Klf4 and c-Myc). Current methods use retroviruses, lentiviruses, adenoviruses or plasmids to maximize cellular transfection and reprogramming. After nuclear reprogramming, the colonies are typically transferred onto fibroblast feeder layers. These cells designated as human induced pluripotent stem cells (iPS) exhibit morphology of embryonic stem cells and express ES cell markers.

present in the somatic cell population or acquired during prolonged reprogramming culture, remains unresolved. Interestingly, in 2007, Yu et al. (31) and Takahashy and Yamanaka (64) carried out astonishing experiments in which they reprogrammed somatic cells into pluripotent stem cells; however, several technical limitations, such as the use of retrovirus or lentiviruses for transfections, restrict the use of such cell lines for clinical applications (67). To help resolve these issues and to make the lines more amenable to clinical applications, methods to induce human iPS cells that leave the genome unaltered are essential and are being actively pursued by several groups.

Both categories of cells (human ES and iPS cells) will find unique uses in the study of stem cell biology and the development and evaluation of therapeutic strategies. Parthenogenesis and somatic cell nuclear transfer (SCNT) represent two major strategies for generating histocompatible hES cells with potential for therapeutic use (68).

CONCLUSION

HES cell lines have been derived from blastocyst ICM cells (16, 69), morulae (2, 5), chromosomally abnormal embryos (70), abnormally developing or arrested embryos (4) and single blastomeres of 8-cell stage embryos (3, 37, 71) and 4-cell stage embryos (72, 38). Although hES cell lines are considered to be very similar in terms of self-renewal, expression of pluripotency markers and the ability to differentiate, it is becoming more evident that differences between lines also exist (73, 74). Marked differences in expression and differentiation potential between these distinct hES cell lines are to be expected, especially because variation between blastocyst ICM-derived hES cell lines has already been described (75). Therefore, the stage of development from which hES cells are derived might be crucial to their use as models of differentiation and in regenerative medicine. In addition, methods for deriving hES cells may affect both differences in hES cell lines and their use in regenerative medicine.

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A CASE OF SEVERE VERAPAMIL INTOXICATION

Vesna Stojanovic Marjanovic[1] ,
Vesna Cokanovic[2]
[1] Emergency Department, Clinical Center "Kragujevac", Kragujevac
[2] Nuclear medicine Department, Clinical Center "Kragujevac", Kragujevac

TEŠKO TROVANJE VERAPAMILOM – USPEŠAN TRETMAN KALCIJUMOM –

Vesna Stojanović Marjanović[1] ,
Vesna Čokanović[2]
[1] Centar za urgentnu medicinu, Klinički centar "Kragujevac", Kragujevac
[2] Centar za nuklearnu medicinu, Klinički centar "Kragujevac", Kragujevac

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ABSTRACT

Introduction: *Verapamil is a potent calcium channel blocker (CCB) used to treat a variety of common cardiovascular abnormalities including hypertension, angina pectoris and supraventricular arrhythmias. The principal effects of verapamil are on the cardiovascular system where it decreases atrioventricular conduction and has a negative inotropic effect.*

Case report: *A 33-year-old woman was admitted to the emergency department due to an intentional overdose of verapamil. She appeared to be lethargic and developed hypotension, A-V dissociation and circulatory failure. She recovered with the appropriate use of symptomatic and supportive therapy.*

The patient received a total of 24 g of calcium gluconate within 24 hours. Smaller amounts of calcium gluconate (1 to 2 g) or larger doses over prolonged periods of time (24 g in 44 hrs) have been administered in other cases described in the literature.

Conclusions: *Large doses of calcium are effective and safe in the treatment of verapamil intoxication when given continuously with adequate monitoring.*

Key words: *intoxication, verapamil, calcium gluconate*

SAŽETAK

Uvod: *Verapamil kao lek iz grupe blokatora kalcijumskih kanala, blokira ulazak kalcijuma u ćeliju. U akutnom trovanju verapamilom se potenciraju njegovi farmakološki efekti na kontraktilnost i sprovođenje u miokardu.*

Prikaz slučaja: *Pacijentkinja stara 33 godine, primljena je u Centar za urgentnu medicinu somnolentna, hipotenzivna, bradikardična sa atrioventrikularnom disocijacijom. Uspešan medikamentozni tretman doveo je do povoljnog ishoda.*

Zaključak: *Navedeni prikaz slučaja smatramo važnim jer u nama dostupnoj literaturi nije opisana primena tako visoke doze kalcijuma u kratkom vremenskom periodu, koja je dala povoljan efekat u trovanju verapamilom.*

Ključne reči: *trovanje, verapamil, kalcijum-glukonat.*

Abbreviations
A-V – Atrio-ventricular
CCB – Calcium channel blocker

Skraćenice
A-V – Atrio-ventrikularni
CCB – Blokatori kalcijumovih kanala

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Correspondence: Vesna Stojanović Marjanović / Daničićeva 26, 34000 Kragujevac
Tel: 034/317-494 / Mob: 064/85-40-620
E-mail: toxicokg@gmail.com

INTRODUCTION

Verapamil belongs to the group of calcium channel blockers. These drugs block the entry of calcium into cells through slow L-type calcium channels during the excitation-contraction phase in smooth muscles and have inhibitory actions on smooth muscles in the heart, blood vessels and the entire heart conducting system. In a normal cell, the concentration of free calcium ion is rather low compared to the concentration in extracellular liquid.

Verapamil is well-absorbed after oral intake, but its bioavailability is only 10-20% because of intense metabolism after its first passage through the liver. With liver insufficiency, the bioavailability goes up to 90%. Approximately 90% of the injected dose is bound to plasma protein. The elimination half time of verapamil is 3 to 8 hours. Norverapamil, a verapamil metabolite, has 20% of the pharmacological activity of verapamil [1,2] and is eliminated primarily through the kidneys. The main effect of verapamil is on conduction in the heart, causing a depression of sinoatrial node and a decrease in atrioventricular conduction. The effect on myocardium and peripheral blood vessels is less pronounced.

With acute verapamil intoxication, the pharmacological effects on contraction and conduction in myocardium are highly emphasised. There is also an effect on the central nervous system. Ingestion of 3 or more grams of verapamil causes serious disorders: conduction disorders in A-V node progressing to A-V dissociation, hypotension, impairment of consciousness, convulsions and breathing disorders. Hyperglycaemia often occurs as a result of decreased insulin release from beta cells in the pancreas, a process dependent on calcium input through slow calcium channels [1]. In treating oral poisonings, gastric lavage is mandatory as well as the administration of activated charcoal. Treatment also includes the administration of 10% calcium chloride or calcium gluconate.

CASE REPORT

A 33-years-old female patient was admitted to the Emergency Department due to verapamil intoxication. The precise circumstances of poisoning are unknown, but family members confirmed an oral intake of verapamil a few hours prior to admittance. She was drowsy, hypotensive (60/40 mmHg), bradycardic (32 beats per minute) and eupneic, with normal appearance and weight. Cardiac action was rhythmic with low tones. Physical examination did not reveal other abnormalities. ECG showed AV block of the third degree with ventricular frequency 32/min. After admission, the patient's stomach was lavaged and activated charcoal was administered. The laboratory analyses showed hyperglycaemia (13.9 mmol/l), leukocytosis (13.ox 109 /l), hypokalemia (3.4 mmol/l) and metabolic acidosis (pH 7.32, HCO3 14.9 mmol/l, BE -11.2 mmol/l). The chest x-ray was normal. The patient was transferred to the intensive care unit and constantly monitored for vital functions. An hour after admittance, the patient's blood pressure rapidly dropped due to asystole, cardiac shock and respiratory arrest. An aggressive therapy, including cardiopulmonary resuscitation, intubation and multiple pharmacological agents, resulted in a positive outcome. In the next two hours, repeated administration sets of calcium gluconate, atropine, adrenaline and dopamine infusions, resulted in normalisation of blood pressure (80/30 mmHg to 100/45 mmHg). Heart frequency varied from 55/min to 60/min.

After five hours, arterial blood pressure and heart frequency normalised and remained stabile until the end of hospitalisation. Sixteen hours later, the endotracheal tube was removed and the patient appeared to be conscious and able to speak. It was revealed later that, in an attempt to kill herself, the patient swallowed 50 tablets of verapamil (80-mg each). During the recovery, ECG showed an AV block of the first degree followed by sinus rhythm with negative T waves in V_2-V_6 and, later, in V_2-V_3.

DISCUSSION

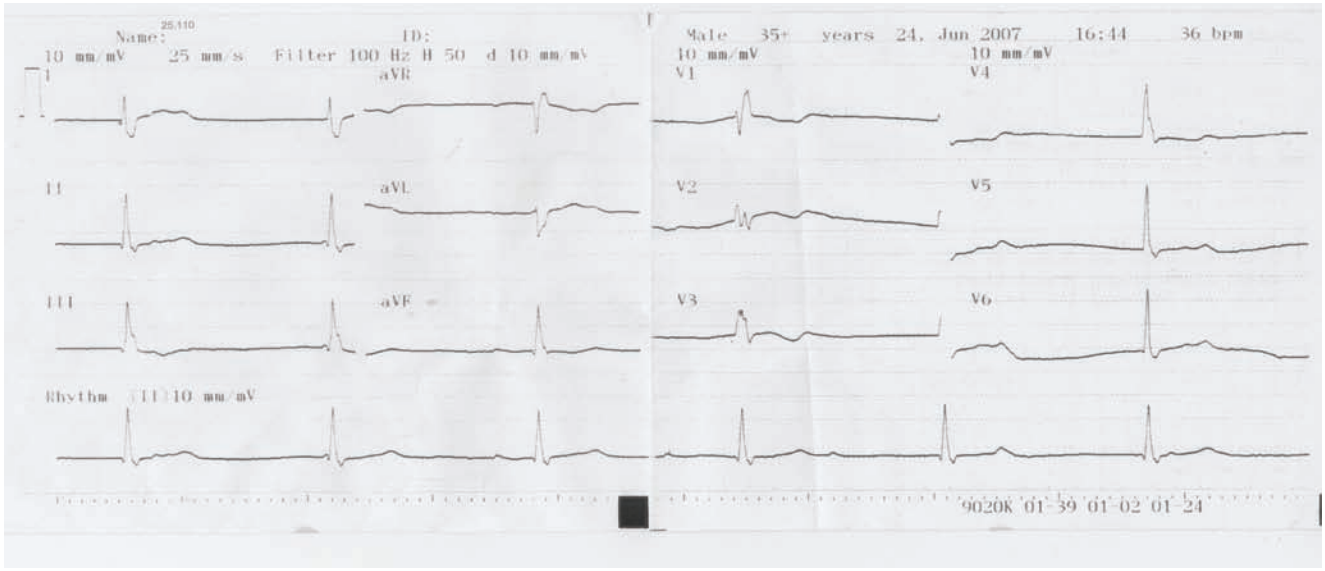
It is always a challenge to treat patients with severe verapamil intoxication. Initial decontamination and supportive measures with intravenous use of calcium and vasoconstrictors are basic principles of treatment. Such a therapy improves haemodynamics and metabolic efficiency. One of the first measures in hypotensive patients is adequate restitution of plasma volume. Gastric lavage and administration of activated charcoal may be considered in patients admitted up to 6 to 8 hours after ingestion [1,3]. This particular patient was admitted 5 hours after the intake of 4 g of verapamil. After admittance, gastric lavage was performed and activated charcoal was administered (1 g/kg BW).

The patient was diagnosed with hypotension and bradycardia at admittance; cardiac shock occurred later. Haemodynamic variables change according to verapamil dose. Approximately 45% of patients who swallow 2 g of verapamil develop hypotension and shock, but 100 % of patients with an intake over 2 g show these same changes. Patients have died after doses of 1.4, 2.4 and 2.8 g, while a recovery was described in a patient who had taken 6 g [4].

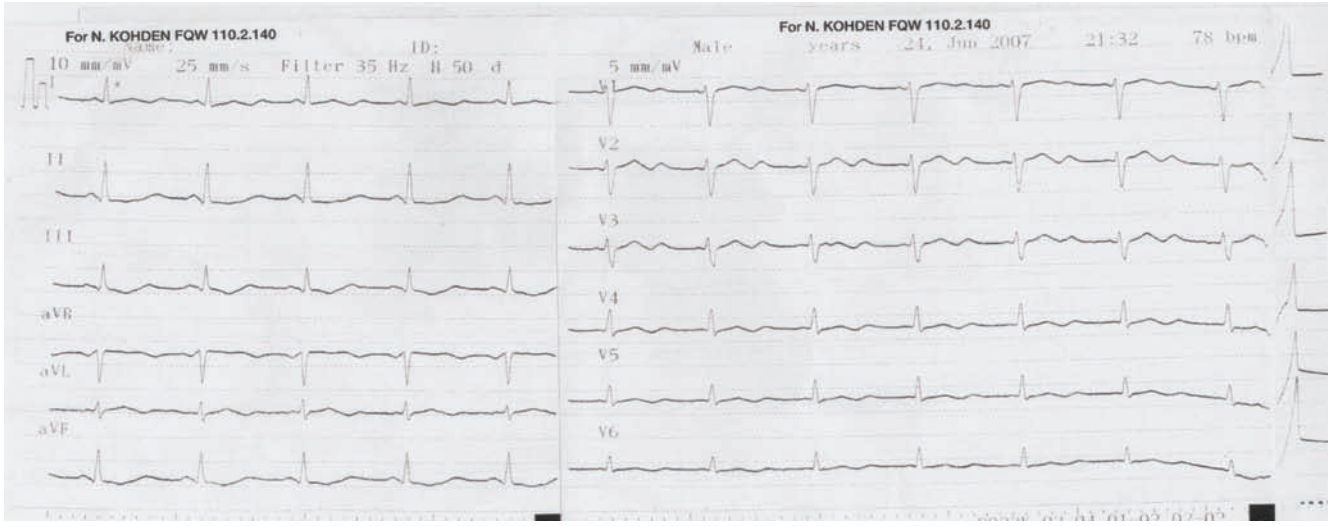
Co-ingestion of verapamil with beta blockers could lead to significant intoxication [5]. The lethal and toxic doses of calcium channel blockers in humans have not been defined precisely. Conclusions on a toxic dose have been approximated from descriptions of intoxication cases [6]. One case described a patient who had taken 9.6 g of verapamil, developed cardiac shock and third-degree A-V block, but recovered after adequate supportive therapy [4].

Due to bradycardia, atropine was given to the patient in repeated doses of 5 mg. Atropine increased heart rate from 55 to 77 beats per minute. However, other studies have shown that atropine neither increases blood pressure nor improves survival[7].

Recoveries have been described after administration of orciprenalin, calcium gluconate or dopamine without an effect on blood pressure. Conversely, adrenaline infusion



ECG on admission



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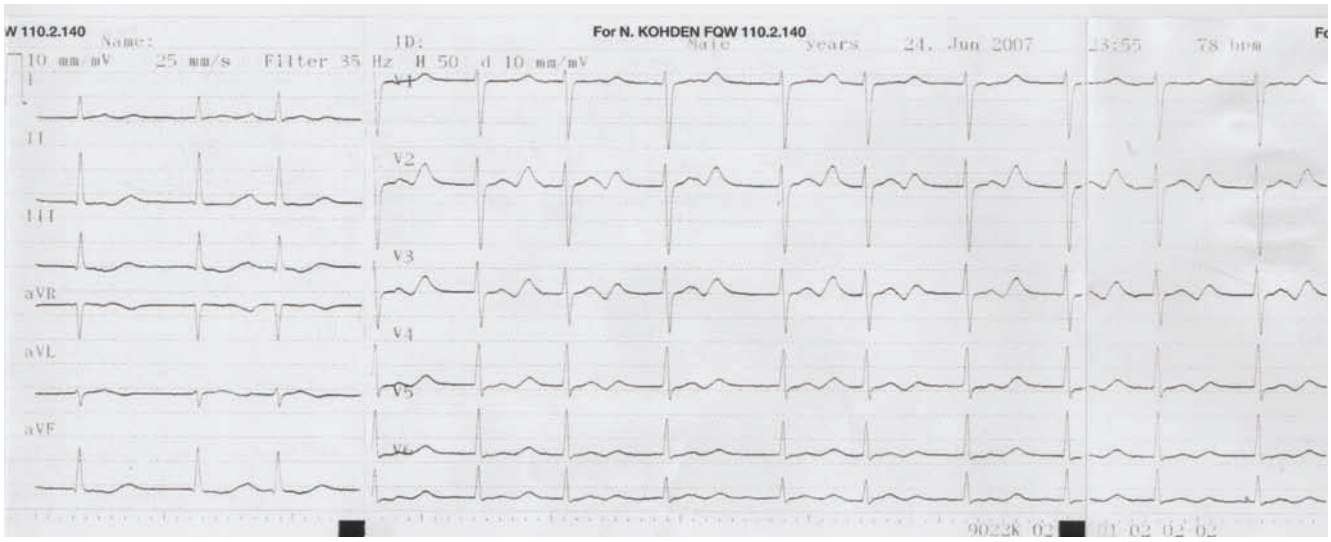

ECG after stabilisation pacient

Figure 1. ECG of the patient intoxicated with verapamil

increases blood pressure and the positive outcome [8]. Animal experiments have shown that adrenaline significantly decreases mortality [7].

In the treatment of our patient, total amounts of 17 mg of adrenaline and 24 g of calcium gluconate with dopamine were administered. Some reference books have described that the administration of 18 g of calcium gluconate within three hours resulted in a positive outcome, while others have used significantly lower (1-2 g) or higher doses, but in longer periods of time (e.g. 24 g in 44 hours) [9]. Some authors suggest concomitant administration of calcium gluconate and NaCl, because of a better exchange of sodium and calcium ions, and the addition of digoxin to atropine[10]. There is no agreement in the literature about optimal dosage of intravenous calcium. Some surveys recommend a dose of 1 g calcium chloride every 15 to 20 minutes up to a total amount of 4 g. Others suggest 1 g every 2-3 minutes up to the maximum effect. The rest claim that the best effects can be achieved by continuous calcium infusion. Recent studies on verapamil intoxication in dogs have shown that the administration of calcium with digoxin had a useful effect on systolic pressure, but monotherapy with calcium was effective only in intoxications with low doses of verapamil[10,11]. Calcium and sympathicomimetic amines are also an effective combination[12].

The atrioventricular block is present in 82% of verapamil intoxication cases, but there is no correlation between ingested dose and the disturbance in atrioventricular conduction. Those could be seen even with ingested doses below 1 g. In 90% of cases, an AV block of the third degree appears, while AV blocks of the first and second degree are present in 10% of cases. A full recovery to the normal sinus rhythm occurs within 5-48 hours [4]. One patient with a severe intoxication experienced hypotension and AV block of the third degree for 48 hours [13].

Atropine is a potentially useful intervention for bradycardia associated with an overdose of calcium-channel blockers. Initial treatment with doses of 0.5 to 1 mg of atropine, every two to three minutes up to a total of 3 mg, may be given. Use of transthoracic and intravenous cardiac pacing to increase heart rate has been successful in patients with an overdose of a calcium-channel blocker. However, two primary limitations have been noted: 1) the failure to capture, and 2) the failure to increase blood pressure, despite successful increases in heart rate. As a last effort in cases in which other treatments have failed, mechanical support with the use of intra-aortic balloon counterpulsation or even extracorporeal bypass should be considered. Because the inotropic failure resulting from an overdose of calcium-channel blockers is generally transient (lasting less than 72 hours), invasive mechanical support and even extracorporeal bypass can be lifesaving and return a patient to his or her previous level of functioning[14].

Cases of CCB-poisoned patients who failed to respond to fluids, calcium, or dopamine and dobutamine, but had significant increases in both heart rate and blood pressure after glucagon administration, have been reported. Glucagon has significant inotropic and chronotropic effects[15].

CONCLUSION

In this case, a favourable effect was reached; a serious acute verapamil poisoning was cured using high doses of calcium gluconate with nonspecific detoxification, symptomatic and supportive therapy. From this case, one can learn that large doses of calcium are an effective and safe treatment of verapamil intoxication when given cautiously and with adequate monitoring.

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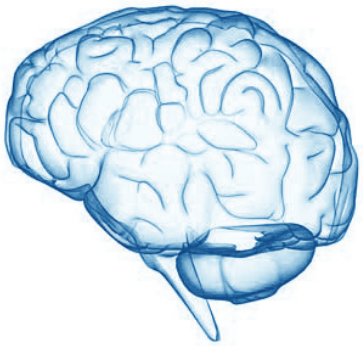

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Svetozara Markovica 69, 34000 Kragujevac, SERBIA
P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
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