FIZIOLOGIA
physiology

ACCREDITED BY CNCSIS - B CATEGORY - CODE 240

Publication data: Fiziologia (Physiology) is issued quarterly

Subscription rates: Subscriptions run a full calendar year. Prices are give per volume, surface postage included.

Personal subscription: Romania - 65 RON, Outside Romania - 35$ (must be in the name of, billed to, and paid by an individual. Order must be marked "personal subscription")

Institutional subscription: 50$ (regular rate)

Single issues and back volumes: Information on availability and prices can be obtained through the Publisher.

Change of address: Both old and new address should be stated and send to the subscription source.

Bibliographic indices: We hope this journal will be regularly listed in bibliographic services, including "Current Contents".

Book Reviews: Books are accepted for review by special agreement.

Advertising: Correspondence and rate requests should be addressed to the Publisher.

1. FOR SUBSCRIPTION ADDRESS

HVB Bank TIMISOARA
RO 21 BACX 0000000218508250

TIMISOARA – ROMANIA
PENTRU REVISTA
„FIZIOLOGIA – PHYSIOLOGY"

2. CORRESPONDENCE SHOULD BE ADDRESSED TO THE CHIEF EDITOR

PROF. DR. FRANCISC SCHNEIDER
PO BOX 135
300024 – TIMISOARA – ROMANIA
e-mail: carmen.tatu@umft.ro

Editura **EUROSTAMPA**
Tel./fax: 0256-204816
ISSN 1223 – 2076

Instructions to Authors

Submission: Only original papers in English are considered and should be sent to:
Prof. dr. Francisc Schneider
Chief Editor of "Fiziologia"
PO Box 135
300024, TIMISOARA, ROMANIA
Tel./Fax: 40-256/490507

Manuscripts should be submitted in triplicate sets of illustrations (of which one is an original), typewritten doublespaced on one side of the paper, with a wide margin.
Conditions: All manuscripts are subject to editorial review. Manuscripts are received with the explicit understanding that they are not under simultaneous consideration by any other publication. Submission of an article for publication implies the transfer of the copyright from the author to the publisher upon acceptance. Accepted papers become the permanent property of "Fiziologia" (Physiology) and may not be reproduced by any means, in whole or in part, without the written consent of the publisher. It is the author's responsibility to obtain permission to reproduce illustrations, tables, etc. from other publications.
Arrangement:
Title page: The first of each paper should indicate the title (main title underlined), the authors' names, and the institute where the work was conducted. A short title for use as running head is also required.
Keywords: for indexing purposes, a list of 3-10 keywords in English and Romanian is essential.
Abstract: Each paper needs abstract and title in Romanian and English language, fonts size 9, Arial Narrow.
Bady text: fonts size 10, Arial Narrow.
Small type: Paragraphs which can or must be set in smaller type (case histories, test methods, etc.) should be indicated with a „p" (petit) in the margin on the left-hand side.
Footnotes: Avoid footnotes. When essential, they are numbered consecutively and typed at the foot of the appropriate page, fonts size 8, Arial Narrow.
Tables and illustrations: Tables (numbered in Roman numerals) and illustrations (numbered in Arabic numerals) should be prepared on separate sheets, fonts size 9, Arial Narrow. Tables require a heading, and figures a legend, also prepared on a separate sheet. For the reproduction of illustrations, only good drawings and original photographs can be accepted; negatives or photocopies cannot be used. When possible, group several illustrations on one block for reproduction (max. size 140x188 mm) or provide crop marks. On the back of each illustration indicate its number, the author's name, and article title. Colour illustration are reproduced at the author's expense.
References: In the text identify references by Arabic figures, (in brackets), fonts size 9, Arial Narrow. Material submitted for publication but not yet accepted should be noted as "unpublished data" and not be included in the reference list. The list of references should include only those publications which are cited in the text. The references should be numbered and arranged alphabetically by the authors' names. The surnames of the authors followed by initials should be given. There should be no punctuation signs other than a comma to separate the authors. When there are more than 3 authors, the names of the 3 only are used, followed by "et al". abbreviate journal names according to the Index Medicus system. (also see International Committee of Medical Journal Editors: Uniform Requirements for manuscripts submitted to biomedical journals. Ann Intern Med 1982; 96: 766 – 771).
Examples:
(a) Papers published in periodicals: Kauffman HF, van der Heide S, Beaumont F, et al: Class-apecific antibody determination against Aspergillus fumigatus by mean of the enzyme-linked immunosorbent assay. III. Comparative study: IgG, IgA, IgM, ELISA titers, precipitating antibodies and IGE biding after fractionation of the antigen. Int Arch Allergy Appl Immunol 1986; 80: 300 – 306.
(b) Monographs; Matthews DE, Farewell VT: *Using and Understanding Medical Statistics*. Basel, Karger, 1985.
(c) Edited books: Hardy WD Jr, Essex M: *FeLV-inducted feline acquired immune deficiency syndrome: A model for human AIDS;* in Klein E(ed): Acquired Immunodeficiency Syndrome. Prog Allergy, Busel, Karger, 1986, vol 37, 353 – 376.
Full address: The exact postal address complete with postal code of the senior author must be given; if correspondence is handled by someone else, indicate this accordingly. Add the E-mail address if possible.
Page charges: There is no page charge for papers of 4 or fewer printed pages (including tables, illustrations and references).
Galley proofs: unless indicated otherwise, galley proofs are sent to the first-named author and should be returned with the least possible delay. Alternations made in galley proofs, other than the corrections of printer's errors, are charged to the author. No page proofs are supplied.
Reprints: Order forms and a price list are sent with the galley proofs. Orders submitted after the issue is printed are subject to considerably higher prices. Allow five weeks from date of publication for delivery of reprints.

FIZIOLOGIA
physiology

CONTENTS

CUPRINS

GENE EXPRESSION OF PROGRAMMED DEATH LIGAND (PD-L)-1, PD-L2, INDUCIBLE COSTIMULATOR LIGAND AND B7-H3 ON HUMAN ALVEOLAR EPITHELIAL CELLS AND REGULATION BY RESPIRATORY SYNCYTIAL VIRUS AND TYPE 1 AND 2 CYTOKINES

AURICA G. TELCIAN[1], ADRIANA MURESAN[2], VASILE LAZA-STANCA[1], SEBASTIAN L. JOHNSTON[1] AND LUMINITA A. STANCIU[1]

[1]Department of Respiratory Medicine, National Heart and Lung Institute, MRC & Asthma UK Centre in Allergic Mechanisms of Asthma and Centre for Respiratory Infection, Imperial College London, Norfolk Place, London W2 1PG, UK

[2]Department of Physiology, University of Medicine and Pharmacy "Iuliu Hatieganul", Cluj-Napoca, Romania

ABSTRACT

Bronchiolitis caused by respiratory syncytial virus (RSV) during infancy is associated with the later development of asthma, and RSV is implicated in exacerbations of asthma. We had shown previously that RSV up-regulates the surface protein expression of co-stimulatory molecules (programmed death-1 ligand [PD-L1], PD-L2, inducible co-stimulator ligand [ICOS-L] and B7-H3) on alveolar epithelial cells. Here, we set up to extend these experiments and to investigate the kinetic of gene expression of these molecules by RSV and the influence of type 1 and type 2 cytokines. Methods. We used quantitative RT-PCR to investigate PD-L1, PD-L2, ICOS-L and B7-H3 gene expression on A549 cells and regulation of this expression by RSV, IFN-γ and IL-4. Results. The messenger RNA for all these co-stimulatory molecules is present on A549 cells and is up-regulated by RSV infection in a replication-specific manner. IFN-γ and IL-4 is differentially modulating the gene expression of PD-L1 and PD-L2 on A549 cells. Conclusion. A549 cells are constitutively expressing a wide range of B7 family molecules, even at messenger RNA level. Knowledge of the expression and function of these co-stimulatory molecules can be used to provide insights into mechanisms of T cell activation and tolerance, with great therapeutic potential for controlling T cell responses.

Key words: B7 co-stimulatory molecules, respiratory syncytial virus, alveolar epithelial cells

INTRODUCTION

The immune system must rapidly respond to diverse microbial pathogens, yet mount a highly specific response that engenders memory of the particular foreign invader. As animal host and pathogenic microbes have coevolved, each in response to other, families of immune system molecules with common structural features have appeared. The co-stimulatory molecules deliver signals that serve to regulate the magnitude and duration of adaptive immune responses. There are several families of co-stimulatory molecules and of the best characterized co-stimulatory pathway involves the B7:CD28 signalling molecules (20).

The programmed death ligand (PD-L)-PD-1 receptor pathway, which is part of the B7:CD28 family, consists of the two ligands PD-L1 and PD-L2 and their common receptor PD-1. Investigators initially identified the PD-1 receptor by subtractive hybridization studies using a T cell hybridoma undergoing programmed cell death, hence its name (8). Subsequent studies demonstrated that PD-1 was expressed on activated T and B cells and was not required for apoptosis (1). PD-Ls expressed on antigen presenting cells interact with their receptor on T cells and regulate primary and memory T cell immune responses (5, 9). The PD-Ls–PD-1 pathway is also important in tolerance and immunopathology (18).

ICOS-L was identified as the third member of the B7 family (2). It is binding to ICOS and delivers a co-stimulatory signal to T cells (proliferative responses and cytokine production). ICOS-L mRNA is expressed constitutively in a variety of lymphoid and nonlymphoid tissues including kidney, liver and peritoneu. The function of ICOS-L expression in nonlymphoid tissue is yet to be elucidated and further studies are required.

One of the latest B7 family members is B7-H3. It is closely related to the ICOS-L but the identity of its counterligand remains to be determined. Although initial studies showed a stimulator role of T cell responses (3), recent studies have indicated that B7-H3 has an inhibitory function (21).

The role of co-stimulatory molecules (PD-L1/2, ICOS-L, B7-H3) in acute viral infections is little studied. Respiratory syncytial virus (RSV) is the major cause of acute bronchiolitis in infants. RSV infects nearly all infants in their first or second winter (7), and though impaired Th1 immunity is implicated (12), it is largely unknown why some infants become seriously ill while the majority have only mild respiratory illness. Immunity to RSV is incomplete and re-infections occur throughout life. The mechanisms for the deficient memory immune response to RSV infection are poorly understood.

Respiratory epithelial cells are the primary site of RSV infection. They express surface molecules associated with antigen presentation (15), including the PD-Ls, ICOS-L and B7-H3 (6, 10, 19). We have previously reported co-stimulatory molecules surface protein expression on respiratory epithelial cell lines and their induction by RSV infection (19). Here we investigated PD-L1, PD-L2, ICOS-L and B7-H3 gene expression in human alveolar epithelial cells (A549) and their regulation by RSV infection and/or type 1 and 2 cytokines.

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Aurica Telcian, Department of Respiratory Medicine, National Heart and Lung Institute, MRC & Asthma UK Centre in Allergic Mechanisms of Asthma and Centre for Respiratory Infection, Imperial College London, Norfolk Place, London W2 1PG, UK

MATERIALS AND METHODS

Cell culture and virus infection

A549 cells, an alveolar adenocarcinoma epithelial cell line (European Collection of Animal and Cell Cultures) were cultured and infected with RSV as previously described (19). RSV A2 strain (gift from Prof. PJ Openshaw, Imperial College London, UK) was grown in Hep-2 cells and virus titre determined by plaque assay.

RNA extraction, reverse transcription, and quantitative PCR

Total RNA was extracted, and 1 µg was used for cDNA synthesis (Qiagen kits). Quantitative PCR was performed using specific primers and probes for PD-L1 (forward primer 5'-GAA TTG GTC ATC CCA GAA CTA CCT-3', reverse primer 5'-GCA TAA TAA GAT GGC TCC CAG AAT-3', probe FAM 5'-TGG CAC ATC CTC CAA ATG AAA GGA CTC A-3'TAMRA), PD-L2 (forward primer 5'- GCT GTG GCA AGT CCT CAT ATC A-3', reverse primer 5'-GCT GCA ATT CCA GGC TCA AC-3', probe FAM 5'-ATA CAG AAC ATG ATC TTC CTC CTG CTA A-3'TAMRA), ICOS-L (forward primer 5'-CAT TGG CTG CTG CAT AGA GAA C-3', reverse primer 5'-AGA CAG GAA ATG ACA TCG GAG AG-3', probe FAM 5'-TCT GCA GCA GAA CCT GAC TGT CG-3' TAMRA), B7-H3 (forward primer 5'-AGC TGT GAG GAG GAG AAT GCA-3', reverse primer 5'-CCA TCA TCT TCT TTG CTG TCA GA-3', probe FAM 5'-CAG CCC TGC AGC CTC TGA AAC A-3'TAMRA) and 18S rRNA (4). Data were analysed using version 1.4 ABI Prism 7500 SDS software, normalized to 18S rRNA and presented as 45-δCT.

Statistical analysis

The results were analysed using GraphPad Prism version 4.00 (GraphPad Software, California). Results of at least three separate experiments were expressed as means±standard errors of the means. When analysing multiple groups, a one way ANOVA of all pairs and columns was used, followed if significant by paired t test for paired comparisons. P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Up-regulation of B7 family molecule gene expression on RSV-infected A549 cells

We previously reported constitutive expression of PD-L1, PD-L2, ICOS-L and B7-H3 surface protein on A549 cells (19). To determine how their gene expression is regulated by RSV infection, time-course experiments were set up and co-stimulatory molecule expression was determined by quantitative RT-PCR (Taqman) (Fig. 1).

A549 cells were cultured up to 72 hours with RSV or UV-inactivated RSV (in order to determine the role of virus replication), harvested at subsequent time-points and Taqman was performed. PD-L1 gene expression was quickly up-regulated by RSV infection, as early as 2 hours post virus infection then remained high, with no big variations, up to 72 hours (Fig. 1A). PD-L2 gene expression was up-regulated by RSV infection with a certain latency compared to PD-L1, but the expression showed a continuous increase up to 72 hours (Fig. 1B). ICOS-L gene expression was also up-regulated by RSV infection at a later time-point and its expression continued to increase up to 72 hours (Fig. 1C), however, B7-H3 mRNA was only slightly up-regulated by RSV infection relative to media treated cells and its expression seemed to be biphasic, with a peak at 8 hours and another one at 48 hours (Fig. 1D).

For all four molecules, the capacity of RSV to amplify their gene expression was virus replication specific, as UV-inactivated RSV had no/small, transiently effect on the gene expression of these molecules on A549 cells (Fig. 1A-D).

On A549 cells, Qian et al (16) also reported the presence of ICOS-L mRNA and the influence of different stimuli like TNF-α, LPS, IFN-γ or IL-4 on ICOS-L gene expression but not in a quantitative manner; here, we report ICOS-L gene expression by using quantitative RT-PCR, and its modulation in the context of virus infection (RSV).

Effects of IFN-γ and IL-4 on RSV induced PD-L1 and PD-L2 gene expression on A549 cells

Differential regulation of PD-L1 / PD-L2 by type 1 (IFN-γ) and type 2 (IL-4) cytokines has been previously demonstrated in different cell types (13). In our

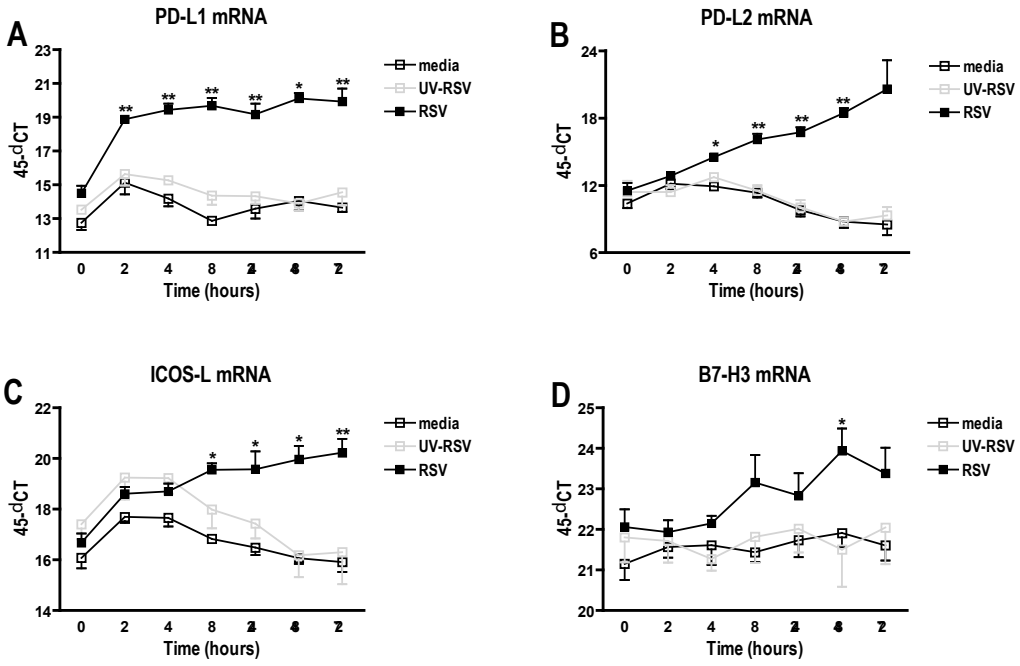


Fig. 1. Respiratory syncytial virus (RSV)-induced PD-L1, PD-L2, ICOS-L and B7-H3 gene expression on A549 cells. Cells cultured in media only (white squares) or infected with RSV (MOI 1, black squares) or UV-inactivated RSV (grey squares) were harvested at the subsequent time-points and PD-L1, PD-L2, ICOS-L and B7-H3 gene expression was measured by quantitative RT-PCR. Shown are the time courses of RSV induction of PD-L1 [A], PD-L2 [B], ICOS-L [C] and B7-H3 [D]. Results are expressed as 45-δCT; data are means±SEs of 3-5 experiments; *p<0.05, **p<0.01 (RSV is compared to cells treated with media).

study, un-infected or RSV-infected A549 cells were cultured with IFN-γ (50ng/mL) or IL-4 (10ng/mL) and mRNA expression for PD-L1 and PD-L2 molecules was

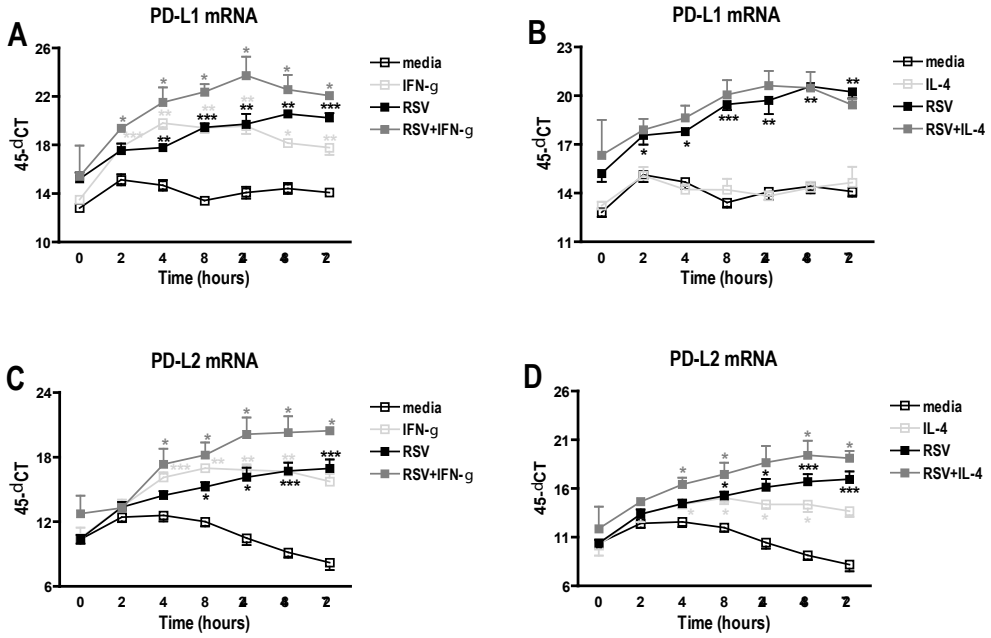


Fig. 2. Regulation by interferon (IFN)-γ and interleukin (IL)-4 of RSV-induced PD-L1 and PD-L2 gene expression on A549 cells. Cells treated with media (white squares) RSV (MOI 1, black squares) with or without either IFN-γ (50ng/mL) or IL-4 (10 ng/mL) (light grey squares correspond to cells treated with the cytokine alone and dark grey squares correspond to cells infected with RSV and treated with cytokines) were harvested at the subsequent time-points and PD-L1 and PD-L2 gene expression was measured by quantitative RT-PCR. Results are expressed as 45-δCT; data are means±SEs of 3-5 experiments; *p<0.05, **p<0.01, ***p<0.001 (RSV and IFN-γ or IL-4 alone are compared to cells treated with media, RSV plus IFN-γ or IL-4 is compared to RSV).

determined. IFN-γ treatment by itself up-regulated on A549 cells PD-L1 and PD-L2 mRNA expression (Fig. 2A and C). Also, IFN-γ treatment had a significant effect on RSV induced PD-L1 and PD-L2 mRNA expression (Fig. 2A and C). IL-4 treatment of A549 cells increased only the expression of PD-L2 mRNA, but not PD-L1 mRNA (Fig. 2B and D). Furthermore, IL-4 treatment of A549 cells up-regulated also RSV-induced PD-L2 mRNA expression (Fig. 2D).

Comparing these results with PD-L1 and PD-L2 surface protein expression on A549 cells and their modulation by IFN-γ and IL-4 (19), it looks like up-regulation of PD-L1 and PD-L2 mRNA by IFN-γ translates quickly into protein and by 24 hours the surface protein is increased as well, in contrast to IL-4 up-regulation of PD-L2 gene expression which doesn't lead to an increased surface protein expression, at least up to 48 hours.

There is increasing evidence that allergic asthma, a chronic airway inflammatory disease characterized by reversible airway obstruction, hyperreactivity, and lymphocyte/eosinophil recruitment, is driven and maintained by chronically activated T cells with a Th2 phenotype. These T cells promote the activation and recruitment of B cells and eosinophils and regulate the Ig class switch to the development of antigen-specific IgE responses (14, 17). Although the significance of B7 molecules expression on respiratory epithelial cells is not fully established, it has been hypothesized that PD-L1, PD-L2, ICOS-L and B7-H3 expression on airway epithelial cells can productively interact with their ligands on T lymphocytes and may play an important role in maintaining or regulating the activation of lymphocytes that have migrated into the respiratory tract, expanding the mechanisms that underlie T cell activation in airway inflammation.

Before developing immunotherapeutic reagents targeting these molecules, dissecting the function of each of the B7 family members in different cells and different settings is important since they can mediate diametric outcomes on T cell mediated immune responses. Therefore reports about co-stimulatory molecule gene or protein expression from different groups on different respiratory epithelial cells (6, 10, 11, 14, 16, 19) are more than welcome in the process of clarifying the function of these molecules.

In conclusion, we showed here that PD-L1, PD-L2, ICOS-L and B7-H3 gene expression is up-regulated by RSV infection. RSV-induced gene-expression of PD-L1 and PD-L2 is differentially regulated by IFN-γ and IL-4. Knowledge of the expression and function of these co-stimulatory molecules can be used to provide insights into mechanisms of T cell activation and tolerance, with great therapeutic potential for controlling T cell responses.

REFERENCES

1. Agata Y, Kawasaki A, Nishimura H, et al: Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996; 8: 765-772
2. Aicher A, Hayden-Ledbetter M, Brady WA, et al: Characterization of human inducible costimulator ligand expression and function. *J Immunol* 2000; 164: 4689-4696
3. Chapoval AI, Ni J, Lau JS, et al: B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol* 2001; 2: 269-274
4. Edwards MR, Johnson MW, and Johnston SL: Combination therapy: Synergistic suppression of virus-induced chemokines in airway epithelial cells. *Am J Respir Cell Mol Biol* 2006; 34: 616-624
5. Greenwald RJ, Freeman GJ, and Sharpe AH: The B7 family revisited. *Annu Rev Immunol* 2005; 23: 515-548
6. Heinecke L, Proud D, Sanders S, et al: Induction of B7-H1 and B7-DC expression on airway epithelial cells by the Toll-like receptor 3 agonist double-stranded RNA and human rhinovirus infection: In vivo and in vitro studies. *J Allergy Clin Immunol* 2008; 121: 1155-1160

7. Hull J, Thomson A, and Kwiatkowski D: Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. *Thorax* 2000; 55: 1023-1027
8. Ishida Y, Agata Y, Shibahara K, et al: Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992; 11: 3887-3895
9. Keir ME, Francisco LM, and Sharpe AH: PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol* 2007; 19: 309-314
10. Kim J, Myers AC, Chen L, et al: Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. *Am J Respir Cell Mol Biol* 2005; 33: 280-289
11. Kurosawa S, Myers AC, Chen L, et al: Expression of the costimulatory molecule B7-H2 (inducible costimulator ligand) by human airway epithelial cells. *Am J Respir Cell Mol Biol* 2003; 28: 563-573
12. Legg JP, Hussain IR, Warner JA, et al: Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. *Am J Respir Crit Care Med* 2003; 168: 633-639
13. Loke P and Allison JP: PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc Natl Acad Sci U S A* 2003; 100: 5336-5341
14. Oei E, Kalb T, Beuria P, et al: Accessory cell function of airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2004; 287: L318-331
15. Papi A, Stanciu LA, Papadopoulos NG, et al: Rhinovirus infection induces major histocompatibility complex class I and costimulatory molecule upregulation on respiratory epithelial cells. *J Infect Dis* 2000; 181: 1780-1784
16. Qian X, Agematsu K, Freeman GJ, et al: The ICOS-ligand B7-H2, expressed on human type II alveolar epithelial cells, plays a role in the pulmonary host defense system. *Eur J Immunol* 2006; 36: 906-918
17. Romagnani S: The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000; 105: 399-408
18. Sharpe AH, Wherry EJ, Ahmed R, et al: The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007; 8: 239-245
19. Stanciu LA, Bellettato CM, Laza-Stanca V, et al: Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines. *J Infect Dis* 2006; 193: 404-412
20. Subudhi SK, Alegre ML, and Fu YX: The balance of immune responses: costimulation verse coinhibition. *J Mol Med* 2005; 83: 193-202
21. Suh WK, Gajewska BU, Okada H, et al: The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat Immunol* 2003; 4: 899-906

EXPRESIA GENELOR PENTRU PROGRAMMED DEATH LIGAND (PD-L)-1, PD-L2, INDUCIBLE COSTIMULATOR LIGAND SI B7-H3 IN CELULELE EPITELIALE ALVEOLARE UMANE SI MODULAREA LOR DE CATRE VIRUSUL SINCITIAL RESPIRATOR SI TIPUL 1 SAU 2 DE CITOKINE

REZUMAT

Virusul respirator sincitial (VRS) cauzeaza bronsiolite in copilarie iar acestea au fost asociate cu dezvoltarea ulterioara a bolii astmatice; in plus, VRS este implicat in exacerbarile astmatice. Este publicat deja modul in care infectia cu VRS amplifica expresia moleculelor co-stimulatori (programmed death-1 ligand [PD-L1], PD-L2, inducible costimulator ligand [ICOSL] si B7-H3) ca proteine de suprafata pe celulele epiteliale alveolare. Obiectivul acestui articol a fost sa investigam expresia genica a aceste molecule co-stimulatori si influenta VRS si tipul 1 sau 2 de citokine. Metode. qRT-PCR (quantitative reverse-transcription polymerase chain reaction) a fost utilizat pentru a investiga expresia genelor pentru PD-L1, PD-L2, ICOS-L si B7-H3 in celulele alveolare A549 si modul in care sunt reglate de catre VRS, IFNγ sau IL-4. Rezultate. Pentru toate aceste molecule ARN-ul mesager este exprimat in celulele A549 in conditii bazale iar nivelul lui este crescut in urma infectiei cu VRS intr-un mod dependent de replicarea virala. IFN-γ sau IL-4 moduleaza in mod diferit expresia genica pentru PD-L1 sau PD-L2 in celulele A549. Concluzii. Celulele A549 exprima in mod constitutiv aceste molecule din familia B7 chiar si la nivelul ARN-ului mesager. Cunoasterea expresiei si functiei acestor molecule co-stimulatori este utila pentru descifrarea mecanismelor in care limfocitele T sunt activate, avand potential terapeutic pentru controlul functiilor limfocitelor T.

Cuvinte cheie: molecule co-stimulatoare B7, virusul respirator sincitial, celule epiteliale alveolare

ASSESMENT OF INTERLEUKIN-1β AND INTERLEUKIN-8 LEVELS IN THE GINGIVAL FLUID OF ORTHODONTIC TRACTIONED TEETH

OLTEANU C.[1], MURESAN A.[2], CRACIUN A.[3], SERBANESCU A.[1], OLTEANU I.[3], KEULARTS MLW IRENE[4]

[1]Department of Orthodontics, „Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca
[2]Department of Physiology, „Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca
[3]Department of Biochemistry, „Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca
[4]Department of Clinical Genetics, Maastricht University, Holland

ABSTRACT

Dental displacement, an obligatory state for a good result of an orthodontic treatment, is obtained by bone resorption and apposition, resulting from an inflammatory process localized at this level. Lymphocytes produce a large number of peptides with regulatory functions upon the organism in autocrine, paracrine or at high distance (endocrine) ways. These peptides were described as cytokines. Among these, interleukin 1β and interleukin 8 are the main pro-inflammatory cytokines, and they can be use as inflammation markers.

In these conditions, the objective of this paper is the assessment of the IL-1β and IL-8 levels in the gingival crevicular fluid of the orthodontic tractioned teeth compared to the teeth upon which no orthodontic force is applied, before as well as after the device application.

The patients taken into this study were 12 girls and 6 boys, between 7 and 12 years, average age of 9.3 years, who exhibited an orthodontic tooth movement of a single tooth (experimental tooth), the homonym tooth from the antagonistic arcade being considered as control. The gingival fluid was sampled before the beginning of the treatment, and after 1 hour, 24 hours and 7 days from the device application. The levels of IL-1β and IL-8 in the gingival crevicular fluid was assessed by ELISA technique.

The level of the two pro-inflammatory interleukins in the gingival fluid of the tractioned teeth was significantly higher ($p<0,01$) at 24 hours from the device application compared with the control teeth; at 7 days from the beginning of the treatment, only the IL-8 level in the experimental teeth being significantly higher compared with the control teeth.

These results demonstrate that the levels of IL-1β and IL-8 in the gingival fluid increase with the dental movement taking place, due to a localized inflammatory process, as a response to mechanical stress.

Key words: interleukin 1β, interleukin 8, gingival fluid, inflammation, orthodontic tractioned teeth

INTRODUCTION

Lymphocytes produce a large number of peptides with regulatory functions upon the organism in autocrine, paracrine or at high distance (endocrine) ways. These peptides were described as cytokines.

Cytokines were first classified in lymphokines, interleukins and chemokines, classification based on their functions, cells involved in their production, and the target for their action. Nowadays the general term of interleukins is used, without other distinctions, because of the pronounced pleiotropism of cytokines.

Until now 35 interleukins (IL) are known, numbered from IL-1 to IL-35 (1). Among these, the pro-inflammatory cytokines are represented by IL-1β, IL-2, IL-6, IL-8, and IL-31.

Interleukin 1β has 153 amino acid residues, and in vivo is mainly produced by monocytes/macrophages. It accomplishes a wide spectrum of stimulatory actions upon the immune cells and upon some cells out of the immune system.

Interleukin 1β is considered as the major mediator of an inflammatory answer (4). It produces immediate, local, inflammatory reactions (edema), and systemic answers as fever or the acute phase response of the liver.

Interleukin 8 has been discovered in 1987 and represents one of the most important mediators of the defensive inflammatory answers (1). Is produced by a very large diversity of cells (monocytes, macrophages, epithelial cells, fibroblasts), hereby any tissue in organism is able to produce this cytokine. IL-1β and TNF α are the major stimuli for IL-8 releasing. IL-8 is released in infections, ischemia, or traumatic injuries associated with high levels of IL-1β and TNF α. The target cells for IL-8 are the neutrophile leucocytes.

Utilization of the orthodontic appliances in the treatment of various dento-maxillar anomalies most frequently suppose the application of some forces of increased intensity, non-physiological, which always will determine an inflammatory answer localized around the tooth or the teeth which are to be displaced. A low-intensity inflammatory answer arisen around the orthodontic displaced tooth is physiologic, simulating the activation of osteoclasts, the apparition of the physiological process of bone remodeling and implicit the tooth displacement by resorption and bone apposition. On the other hand, a high-intensity inflammatory answer could produce the apparition of some pathological phenomena, either localized around the displaced tooth, or generalized at the level of the all oral cavity.

The biologic active substances, such as cytokines and enzymes, are released

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Cristi Olteanu, Department of Orthodontics, „Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, e-mail: cristidolteanu@yahoo.com

by cells in periodontal tissue as an answer to the mechanical stress produced by the orthodontic appliances (3, 9). The purposes of many researches were to better understand the way by which the mechanical stress produced by orthodontic devices determine an answer from the periodontal cells. A non-invasive modality to asses the level of these active substances is the determination of their concentration in the gingival fluid (6, 7).

A series of studies have proven changes in the interleukine-1β and IL-8 concentrations in the gingival fluid of the teeth upon which orthodontic forces are exerted (7, 2, 12).

Thus, the objective of this paper is the assessment of the IL-1β and IL-8 levels in the gingival crevicular fluid of the orthodontic tractioned teeth compared to the teeth upon which no orthodontic force is applied, before as well as after 1 hour, 24 hours, and 7 days after the device application.

SUBJECTS AND METHODS

The written consent of the parents of the child patients taken into this study was obtained after a previous description of the study's protocol. In order to asses the interleukin levels, 18 patients were taken into study, in which 288 measurements were performed. The 18 patients, 12 girls and 6 boys (between 7 and 12 years, average age of 9.3 years), displayed the following criteria for including in study:

- Well general status, without known affections;
- Lack of antibiotic therapy within the last 6 months;
- Absence of anti-inflammatory drugs administration in the month before the study;
- Healthy periodontal tissues and appropriate oral hygiene.

In each patient a brief instruction for teeth brushing and oral hygiene was made before the beginning of the orthodontic treatment. The tooth on which the orthodontic force has been applied was considered as experimental tooth, and the homonym tooth on the antagonistic arcade was considered control tooth (i.e. superior left canine/inferior left canine). The homonym teeth from the antagonistic arcade were not exposed to any orthodontic force.

The orthodontic treatment in the case of all the 18 patients has been achieved using biomechanical appliances. The level of the dental displacement during the 7 days of the study has been measured on the working models, after taking the impressions.

Samples of gingival crevicular fluid (for the experimental tooth, and control tooth respectively) have been collected after the following time intervals:

- Moment 0 – before the initiation of the orthodontic treatment;
- Moment 1 – at 1 hour from the device application;
- Moment 2 – at 24 hours from the device application;
- Moment 3 – at 7 days from the device application.

The sampling of the gingival fluid was made as follows: the teeth were gently washed with a water spray, then isolated with cotton bullets, and finally gently dried with the air spray. Paper cones were used for sampling (Absorbent paper points – Meta). A first cone was introduced in the gingival crevice, maintained here a minute, then a second cone was introduced in the same place. Cones contaminated with blood or saliva have been discarded.

The paper cones were introduced in Eppendorf tubes and kept at -80°C until the measurements were performed. Before the experimental study was achieved, over the cones 200 µl physiologic serum was added, then a centrifugation at 3600 rpm for 10 minutes was performed. The resulted supernatant was utilized for the determination of interleukin and of total protein concentrations.

The levels of IL-1β and IL-8 in the gingival crevicular fluid was assessed by ELISA technique (Quantikine, R&D Systems Inc., Minneapolis, Minnesota, USA), the results being exprimed as pg/µg total proteins in the gingival fluid. The level of total proteins was assessed by Bradford method, using as a standard a BSA (Bovine Serum Albumine) solution.

Concerning the statistical analysis, in order to compare the mean values Student t-test was utilised, because the values have displaied a normal distribution. The correlations between variables were determined with the Spearman test. The statistical analysis was accomplished with the Statistical Package for Social Sciences (SPSS), and with the statistical software of Microsoft Office.

RESULTS

The average level of the dental displacements was of 1.5±0.3 mm, after the 7 days of the study. No dental displacement was observed for the control teeth at any subjects involved in this study. The mean concentration of interleukins 1β and 8 in the gingival crevicular fluid of the experimental and control teeth for the patients involved in study are emphasized in table I.

Table I. The mean concentration of interleukins 1β and 8 in the gingival crevicular fluid of the experimental and control teeth of the patients involved in the study (pg/µg total protein)

	MOMENT							
	ZERO		*1 HOUR*		*24 HOURS*		*7 DAYS*	
	Control	Experim.	Control	Experim.	Control	Experim.	Control	Experim.
IL-1β	0.216	0.223	0.222	0.267	0.219	0.571	0.214	0.221
IL-8	114.9	114.3	112.6	118.4	116.7	350.7	114.9	328.8

The variations of the average values of IL 1β and IL 8 at the time intervals considered are presented in the Figures 1 and 2.

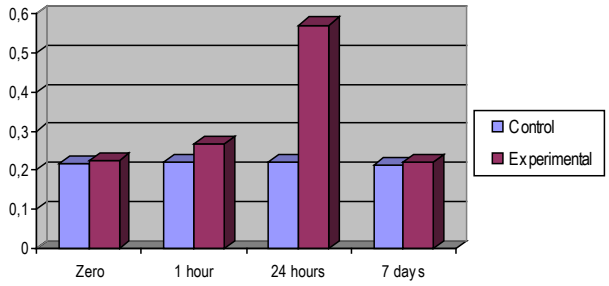


Fig. 1. Modifications of IL-1β concentration in the gingival liquid during the orthodontic treatment (pg/µg)

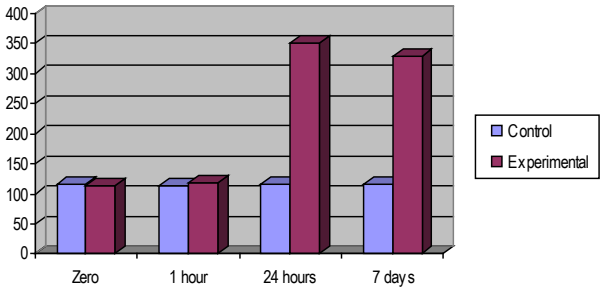


Fig. 2. Modifications of IL-8 concentration in the gingival liquid during the orthodontic treatment (pg/µg)

At moment 0 (before application of the device), the IL-1β level for the experimental tooth, and control respectively was having close values (0.216 pg/µg, and

0.223 pg/µg respectively), the difference being statistically insignificant ($p>0.5$). The same statistically insignificant difference ($p>0.5$) was also recorded at moment 0 for IL-8 (114.3 pg/µg in case teeth, 114.9 pg/µg in control teeth).

At 1 hour after the beginning of the force application, the values of the two interleukins in the gingival fluid of the moved teeth (experimental) have slightly increased levels, but statistically insignificant (1,2 times higher for IL-1β, and 1,04 times higher for IL-8). The concentrations of the two interleukins in control teeth range in the same limits as in moment 0.

At 24 hours from the orthodontic device application, a pronounced increase, statistically significant ($p<0.01$), of the two interleukins levels has been observed in the experimental teeth (2.57 times for IL-1β, and 3.07 times for IL-8), while these levels are keeping relative constant in the control teeth. In control teeth no modifications are noticed in the interleukins' concentration as compared to moment 0.

At 7 days since the treatment has begun, the behavior of the two interleukins diversifies:

- IL-1β levels returns to the initial values (0.221 pg/µg as compared with 0.223 pg/µg at moment 0)
- Il-8 level keeps increased (328.8 pg/µg as compared to 114.3 pg/µg at moment 0), but decreases as compared to the maximal level reached at 24 hours – 350.7 pg/µg – the decreasing being statistically insignificant.

Another result of this study was the achievement of a statistically significant correlation between the levels of the two interleukins before the orthodontic device application and at 24 hours from the action of the orthodontic force.

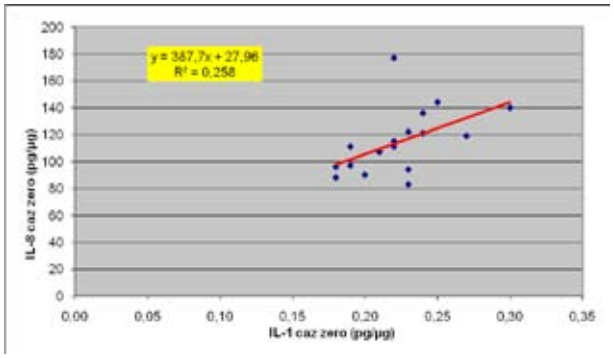


Fig. 3. Correlation between the IL-1β and IL-8 levels in the experimental teeth before the beginning of the treatment

DISCUSSION

In this paper we proved the existence of an IL-8 level approximately 500 times higher as compared to those of the IL-1β in the gingival liquid at moment 0. These values reported in our study are in correspondence with the data in literature (5,11).

The effect of application of a force upon the periodontal ligaments is rapid. Thus, the cells localized at this level respond by releasing of modulators after several minutes since the force was applied, as proved by Storey (10) on incisors guinea pigs. In this study, the two interleukins have a slightly increased level at 1 hour from the beginning of the force application, as compared to moment 0, confirming the literature data. Because of the application of the orthodontic force, an inflammatory process of reduced intensity arises at 1 hour from the device application. This will stimulate the resorption, and bone apposition processes necessary for the dental displacements.

The fact that the IL-1β level has increased faster than the IL-8 level denotes that IL-1β is the first of those taken into study to indicate the initiation of an inflammatory process.

At 24 hours from the orthodontic device application, in experimental teeth a marked increase of the concentration of the two interleukins can be observed (2.57 times for IL-1β, and 3.07 times for IL-8), and which is statistically significant ($p<0.01$). A series of other studies have proved similar results, such as the reaching of a maximal concentration of the pro-inflammatory interleukins at 24 hours from the moment when the force was first applied (2,7,12,13).

Because the pro-inflammatory interleukins are markers of inflammation, the statistically significant increase of their level in the gingival fluid of the orthodontic tractioned tooth at 24 hours from the debut of the treatment denotes the initiation of a localized inflammatory process, inflammation strictly requested for dental displacements. The pain sensation at 24-48 hours after the moment of orthodontic device application is a clinical sign of this process.

At 7 days from the treatment beginning, the behavior of the two interleukins is different:

- The level of IL-1β returns to the initial values (0.221 pg/µg as compared to 0.223 pg/µg at moment 0)
- The level of IL-8 maintains increased (328.8 pg/µg as compared to 114.3 pg/µg at moment 0), but decreases as compared to the maximal level reached at 24 hours – 350.7 pg/µg – a decrease which is statistically insignificant.

The faster return to the initial values of IL-1β as compared to those of IL-8 could be explained by the fact that IL-1β is essential for IL-8 induction, probable via an autocrine, or paracrine mechanism (Maeda et al., 2007) (8).

Because in this study we used biomechanical orthodontic devices, and which were no activated in this period of time, the returns of IL-1β concentration to the initial values after 7 days denotes the absence of the inflammatory process due to the cessation of the bone remodeling process. These phenomena will restart after a new activation of the device.

CONCLUSIONS

1. The application of an orthodontic appliance is followed by an inflammatory process localized strictly around the tooth or the teeth orthodontic tractioned.

2. The localized inflammatory process secondary arisen to the action of the orthodontic force is necessary for the tooth displacement, in order to ensure the bone remodeling by resorption and apposition.

3. The inflammatory process, localized around the tooth upon which the orthodontic force operates, is manifested at 1 hour since the force application, is maxim at 24 hours, then at 7 days reduces near to disappearance.

4. These phenomena will repeat at a new activation of the orthodontic device.

5. The studied markers, which demonstrate the existence of this inflammatory process and to whose variation is statistically and clinically significant, are IL-1β, and IL-8, sampled from the gingival fluid of the orthodontic tractioned tooth.

REFERENCES

1. Alaverdi N, Sehy D. Cytokines – master regulators of the immune system. URL http://www.ebioscience.com/ebioscience/whatsnew/pdf/cytokines.pdf [cited february 17, 2008]
2. Barsan G, Ozer T, Kaya FA. Interleukins 2,6 and 8 levels in human gingival sulcus during orthodontic treatment. *Am J Orthod Dentofac Orthop*. Jul 2006; 130(1): 71-6
3. Davidovitch Z, Nicolay OR, Ngan PW, Shanfeld JL. Neurotransmitters, cytokines, and the control of alveolar bone remodeling in orthodontics. *Dent Clin North Am*. 1988; 32: 411-35
4. Dinarello CA. Interleukin-1 and its biologically related cytokines. Adv Immun. 1989; 44: 153-205

5. Giannopoulou C, Mombelli A, Tsinidou K, Vasdekis V, Kamma J. Detection of gingival crevicular fluid cytokines in children and adolescents with and without fixed orthodontic appliances. *Acta Odontol Scand*. 2008 Jun; 66(3): 169-73.
6. Griffiths GS, Moulson AM, Petrie A, James IT. Evaluation of osteocalcin and pyridinium crosslinks of bone collagen as markers of bone turnover in gingival crevicular fluid during different stages of orthodontic treatment. *J Clin Period.* 1998; 25: 492-98
7. Iwasaki LR, Haack JE, Nickel JC, Reinhardt RA, Petro TM. Human interleukin-1 beta and interleukin-1 receptor antagonist secretion and velocity of tooth movement. *Arch Oral Biol.* 2001; 46: 185-89
8. Maeda A, Soejima K, Bandow K, Kuroe K, Kakimoto K, Miyawaki S et al. Force-induced IL-8 from periodontal ligament cells requires IL-1beta. *J Dent Res.* 2007 Jul; 86(7): 629-34.
9. Saito M, Saito S, Ngan PW, Shanfeld JL, Davidovitch Z. Interleukin-1β and prostaglandin E are involved in the response of periodontal cells to mechanical stress in vivo and in vitro. *Am J Orthod Dentofac Orthop.* 1991; 99: 226-40
10. Storey E. The nature of tooth movement. *Am J Orthod.* 1973; 63: 292-314
11. Tuncer BB, Ozmeriç N, Tuncer C, Teoman I, Cakilci B, Yücel A, Alpar R, Baloş K. Levels of interleukin-8 during tooth movement. *Angle Orthod.* 2005 Jul; 75(4): 631-6.
12. Uematsu S, Mogi M Interleukin (IL)-1 β, IL-6, TNF-α, epidermal growth factor and beta-2 microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movement *J Dent Res.* 1996 Jan; 75(1): 562-7
13. Yamaguchi M, Yoshii M, Kasai K. Relationship between substance P and interleukin-1β in gingival crevicular fluid during orthodontic tooth movement in adults. *Eur J Orthod.* 2006 Jun; 28(3): 241-46

DETERMINAREA NIVELULUI INTERLEUKINEI-1β ŞI INTERLEUKINEI-8 ÎN LICHIDUL GINGIVAL AL DINȚILOR TRACȚIONAȚI ORTODONTIC

REZUMAT

Deplasarea dentară, condiție obligatorie pentru reuşita unui tratament ortodontic, se realizează prin procese de resorbție şi apoziție osoasă, apărute secundar existenței unui proces inflamator localizat la acest nivel. Limfocitele produc numeroase peptide care în mod autocrin, paracrin sau la distanță mare (endocrin) exercită funcții reglatoare asupra organismului. Aceste peptide au fost descrise ca citokine. Dintre acestea, interleukina-1β şi interleukina-8 reprezintă principalele citokine cu rol proinflamator, putând fi astfel utilizate ca markeri ai inflamației.

În aceste condiții, obiectivul lucrării îl constituie determinarea nivelului IL-1β şi IL-8 în lichidul gingival crevicular al dinților tracționați ortodontic, comparativ cu dinții asupra cărora nu se exercită nicio forță ortodontică, înainte şi după aplicarea aparatului.

Pacienții cuprinşi în studiu au fost 12 fete şi 6 băieți, cu vârsta medie 9,3 ani, care au prezentat o deplasare în scop ortodontic a unui singur dinte (dintele caz), dintele omonim de pe hemiarcada antagonistă fiind considerat dinte martor. Lichidul gingival a fost recoltat atât de la dintele caz, cât şi de la dintele martor, înainte de debutul tratamentului, la 1 oră, 24 ore şi 7 zile de la debutul deplasării dentare. Nivelul interleukinelor în lichidul gingival a fost determinat utilizând un kit ELISA.

Concentrația celor două interleukine în lichidul gingival al dinților tracționați ortodontic a fost semnificativ crescută ($p<0,01$) la 24 ore de la aplicarea forței comparativ cu dinții martor, la 7 zile de la debutul tratamentului ortodontic, doar concentrația interleukinei-8 la dinții caz fiind semnificativ crescută comparativ cu dinții martor ($p<0,01$).

Aceste rezultate demonstrează că nivelul IL-1β şi IL-8 în lichidul gingival creşte odată cu desfăşurarea deplasărilor dentare, creştere datorată apariției unui proces inflamator localizat, ca răspuns la existența unui stres mecanic.

Key words: interleukina 1β, interleukina 8, fluid gingival, inflamație, tracționare ortodontică

RESISTANCE TO BETA-LACTAM ANTIBIOTICS OF ESCHERICHIA COLI STRAINS ISOLATED FROM UROCULTURES

DELIA BERCEANU VADUVA, MONICA LICKER, DELIA MUNTEAN, DANA VELIMIROVICI, MATILDA RADULESCU, DUGAESESCU DORINA, CARMEN TATU, MARCELA ADAMUT, STANGA LIVIA, MARIA RADA, M. BERCEANU VADUVA, ROXANA MOLDOVAN

University of Medicine and Pharmacy"Victor Babeş"Timişoara

ABSTRACT

Purpose: Considering the high incidence of urinary infections we made a study in order to determine the antibiotic susceptibility to beta-lactams of Escherichia coli (E. coli) strains isolated from urocultures of patients admitted in the urology department, as well as from ambulatory patients.

Material and methods: We studied 526 strains of E. coli (350 from ambulatory patients and 176 from the urology department).

The germs were identified by the API method (BioMerieux) and the antibiotic susceptibility was performed by disk-diffusion Kirby-Bauer tests. Statistic analysis of the antibiograms and the determination of the resistance phenotypes were performed with automatic reading methods (Osiris – Bio Rad Laboratories). For identifying ESBL (beta-lactamase with extended spectrum) producing strains, the sinergy tests were also performed.

Results: The wild phenotype was present in 49.15% of the strains isolated from ambulatory patients, and only in 17.04% in patients admitted in the urology department. In ambulatory patients, as well as in patients admitted in the urology department, most of the strains were penicilinase-producing (46.85%, respectively 57.95%).

Conclusions: We observed a constant lowering of the sensitivity of E. coli strains to beta-lactams, especially in the urology department, which draws attention to the importance of monitoring antibiotics prescriptions.

Keywords: Escherichia coli, urocultures, resistance phenotype

PURPOSE

Considering the high incidence of urinary infections, in order to prevent selection of multiresistant bacterial strains and to choose the best drug therapy, we made a study to determine the antibiotic susceptibility to beta-lactam of E. coli strains isolated from urocultures of patients admitted in the urology department, and from ambulatory patients, as well as to establish the resistance phenotype of these strains.

MATERIAL AND METHODS

We studied 526 strains of E. coli (350 from ambulatory patients and 176 from the urology department).

The germs were identified by the API method (BioMerieux) and the antibiotic susceptibility was performed by disk-diffusion Kirby-Bauer tests. Statistic analysis of the antibiograms and and the determination of the resistance phenotypes were performed with automatic reading methods (Osiris - Bio Rad Laboratories).

For identifying ESBL (beta-lactamase with extended spectrum) producing strains, the sinergy tests were also performed.

In order to test the sensitivity to beta-lactam antibiotics the following drugs were used: ampicillin (AMP), amoxicillin + clavulanic acid (AMC), ticarcillin (TIC), cephalothin (CF), cefuroxime (CXM), ceftazidime (CAZ).

RESULTS AND DISCUSSIONS

We studied the 526 strains of E. coli isolated from 3760 urocultures:

- from 1889 ambulatory urocultures, 431 were positive and only 350 were E. coli;
- from 1871 urocultures obtained from urology sections, 462 were positive and only 176 were E. coli

Table I and Figure 1 show the distribution of urocultures in the urology department and ambulatory patients.

Table nr. I: Distribution of urocultures in the urology department and ambulatory patients

	Positive urocultures	Sterile urocultures
Ambulatory	431	1458
Urology	462	1409
Total	**893**	**2867**

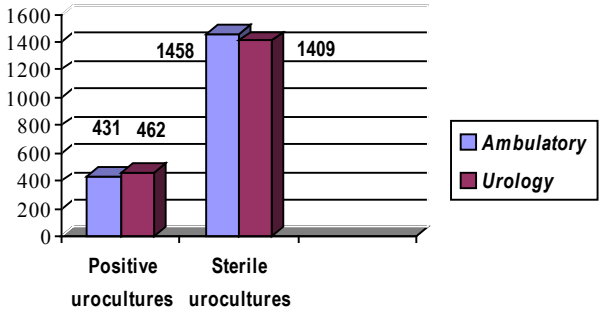


Fig. 1. Distribution of urocultures in the urology department and ambulatory patients

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Delia Berceanu, University of Medicine and Pharmacy"Victor Babeş"Timişoara, 2A Eftimie Murgu Square, 300041, Timisoara, Romania, e-mail: deliaberceanu@yahoo.com

Tables II and III show the distribution of species isolated from the positive urocultures (ambulatory and urology).

Table II. Distribution of species isolated from the positive urocultures - ambulatory

Germs	Frequency	Percent
Escherichia coli	**350**	**81.21%**
Klebsiella pneumoniae	36	8.35%
Proteus mirabilis	14	3.24%
Enterococcus spp.	10	2.32%
Pseudomonas aeruginosa	4	0.93%
Proteus vulgaris	3	0.69%
Enterobacter cloacae	3	0.69%
Staphylococcus aureus	3	0.69%
coagulase-negative staphylococci	2	0.46%
Citrobacter freundii	2	0.46%
Streptococcus agalactiae	2	0.46%
Morganella morganii	1	0.23%
Acinetobacter baumannii	1	0.23%
Total	**431**	**100%**

Table III. Distribution of species isolated from the positive urocultures - urology

Resistance to beta-lactams antibiotics of isolated E. coli strains is shown in Tables IV and V, as well as in Figure 2.

Germs	Frequency	Percent
Escherichia coli	**176**	**37.76%**
Klebsiella pneumoniae	119	25.53%
Enterococcus faecalis	23	4.93%
Pseudomonas aeruginosa	23	4.93%
Streptococcus agalactiae	21	4.5%
Staphylococcus aureus	19	4.07%
Serratia marcescens	18	3.86%
Proteus mirabilis	12	2.57%
Enterobacter cloacae	12	2.57%
Enterobacter gergoviae	12	2.57%
Staphylococcus saprophyticus	9	1.93%
Acinetobacter baumannii	5	1.07%
Staphylococcus haemolyticus	3	0.64%
Stenotrophomnas maltophilia	2	0.42%
Citrobacter freundii	2	0.42%
Citrobacter koseri	2	0.42%
Serratia liquefaciens	2	0.42%
Serratia odorifera	1	0.21%
Klebsiella oxytoca	1	0.21%
Providencia stuartii	1	0.21%
Staphylococcus epidermidis	1	0.21%
Pseudomonas fluorescens	1	0.21%
Aeromonas hydrophila	1	0.21%
Total	**466**	**100%**

Table IV. Resistance to beta-lactams antibiotics of E. coli strains - ambulatory

ANTIBIOTIC	SENSITIVE		INTERMEDIATE		RESISTANT	
	Nr.	%	Nr.	%	Nr.	%
AMPICILLIN	162	46.29%	23	6.57%	165	47.14%
AMOXICILLIN + CLAVULANIC ACID	311	88.86%	39	11.14%	-	-
TICARCILLIN	185	52.86%	-	-	165	47.14%
CEPHALOTIN	308	88.00%	18	5.14%	24	6.86%
CEFUROXIME	345	98.57%	-	-	5	1.43%
CEFTAZIDIME	349	99.71%	-	-	1	0.69%

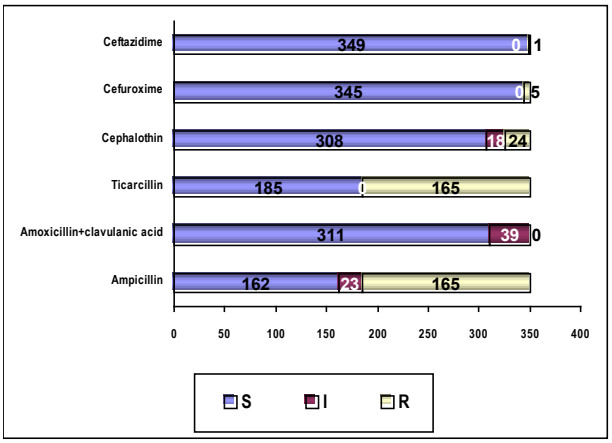


Fig.2. Resistance to beta-lactams antibiotics of E. coli strains - ambulatory

Table V. Resistance to beta-lactams antibiotics of E. coli strains - urology

ANTIBIOTIC	SENSITIVE		INTERMEDIATE		RESISTANT	
	Nr.	%	Nr.	%	Nr.	%
AMPICILLIN	30	17.04	-	-	146	82.95
AMOXICILLIN + CLAVULANIC ACID	68	38.63	2	1.13	106	60.22
TICARCILLIN	30	17.04	-	-	146	82.95
CEPHALOTIN	30	17.04	38	21.59	108	61.36
CEFUROXIME	68	38.63	-	-	108	61.36
CEFTAZIDIME	132	75	-	-	44	25

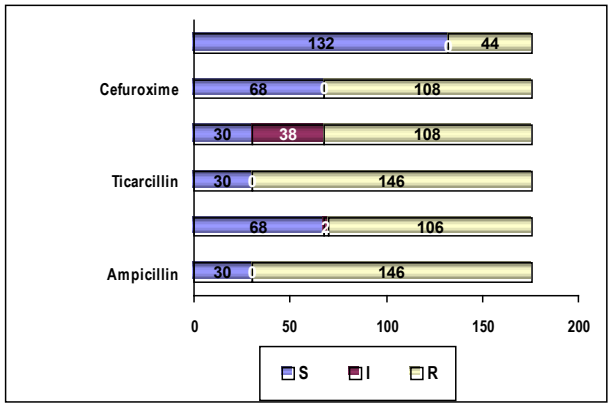


Fig. 3. Resistance to beta-lactams antibiotics of E. coli strains - urology

The interpretative analysis of the antibiograms allowed establishing of the resistance phenotypes of E. coli strains. Through the determination of the resistance phenotypes to antibiotics, information on the resistance mechanisms could be obtained. Based on these mechanisms, the antibiotics to which the tested bacteria was resistant could be detected.

Table VI and Figure 4 show the resistance phenotypes to beta-lactams of E. coli strains isolated from patients from the urology department and the ambulatory.

Table VI. Resistant phenotypes of E. coli to beta-lactams

Phenotype	Ambulatory		Urology	
	Nr.	%	Nr.	%
Wild phenotype (sensitive phenotype)	172	49.15	30	17.04
Low PASE	128	36.57	38	21.59
High PASE	36	10.28	64	36.36
CASE	4	1.13	-	-
ESBL	1	0.29	40	22.72
CHN	-	-	4	2.27

Legend: PASE – penicillinase, CASE – cephalosporinase, ESBL - beta-lactamase with extended spectrum, CHN - quinolone

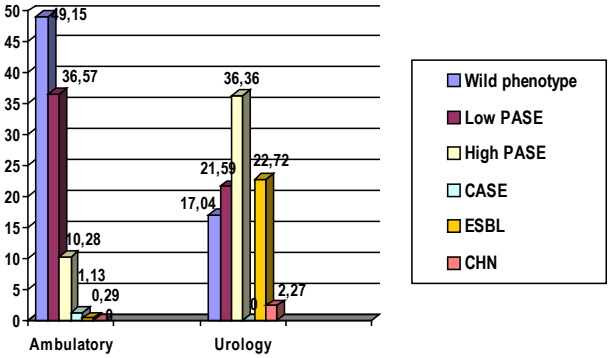


Fig. 4. Resistant phenotypes of E. coli to beta-lactams

The wild phenotype was present in 49.15% of the strains isolated from ambulatory patients, and only in 17.04% in patients admitted in the urology department. In ambulatory patients, as well as in patients admitted in the urology department, most of the strains were penicilinase-producing (46.85%, respectively 57.95%).

A warning sign is the presence of E. coli strains producing beta-lactamase with extended spectrum (ESBL), multi-resistant strains that are usually isolated in hospital environments. This can be explained by the empyrical, irrational and abusive use of antibiotics without an antibiogram.

ESBL strains were predominant in the urology department (22.27%), with only 0.29% in the ambulatory.

CONCLUSIONS

1. We studied 526 strains of E. coli (350 from ambulatory patients and 176 from the urology department) isolated from 3760 urocultures.

2. The purpose of the study was to test the sensitivity to beta-lactams antibiotics of E. coli strains isolated from different ambulatory specimens, as well as to establish the resistance phenotypes of these strains, in order to choose the best chemotherapeutic agent and avoid multi-resistant bacterial strains.

3. We observed a constant lowering of the sensitivity of E. coli strains to beta-lactams, especially in the urology department, which draws attention to the importance of monitoring antibiotic prescriptions.

4. We consider that the development of resistance to antibiotics of these germs is due to the antibiotic empyrical treatments, without an antibiogram. The abusive, irrational and long-term treatment with antibiotics led to the selection of multi-resistant strains, as well as to the creation of an unbalance of saprophytic organisms and dissemination of the pathogenic and facultative pathogenic ones.

5. An alarm signal is raised by the presence of multiresistant ESBL E. coli strains, most frequently isolated from the urology department (22.72%), compared to only 0.29% in ambulatory patients.

6. In establishing a correct anti-infectious therapy, the role of the microbiology laboratory is to perform the antibiogram, as well as to establish the resistance phenotypes, thus offering information on the development of the resistance mechanisms and the ways the phenotypes may spread.

REFERENCES

1. Andronescu D, Olteanu D. Pielonephryta. *Ed. Medicala*, Bucharest, 1996.
2. Angelescu M. Antibiotics therapy. *Ed. Medicala*, Bucharest, 1998.
3. Berceanu Vaduva D et al. Nosocomial infections. *Ed. Mirton*, Timisoara, 1999.
4. Buiuc D, Negut M. Clinical microbiology book 2nd edition. *Ed. Medicala*, Bucharest, 2008.
5. Gluhovschi G, Dragan I et al. Urinary tract infections in medical practice. *Ed. Helicon*, Timisoara, 1992.
6. Greenwood D. *Antimicrobial Chemotherapy*, Oxford, University Press, 1989.
7. Jacoby G, Medeiros AA. More extended-spectrum beta-lactamases. *J. Antimocrob. Agents Chemother* 1991; 35, 1697-1704.
8. Jehl F, Chomarat M, Weber M, Gerard A. From antibiogram to prescription. *Ed. Stiintelor Medicale*, Bucharest, 2004.
9. Licker M, Moldovan R et al. Special microbiology volume I - Bacteriology. *Ed. Eurostampa*, Timisoara, 2008.
10. Licker M, Moldovan R et al. Antibiotics resistence, history and actuality. *Ed. Eurostampa*, Timişoara, 2002.
11. Moldovan R et al. Microbiology – Practical laboratory support, lito UMFT, 2002.
12. Moldovan R al. Medical microbiology volume II, lito UMFT, 2005.
13. Philippon A, Labia R, Jacoby G. Extended-spectrum beta-lactamases. *J. Antimicrob. Agents Chemother*1989; 33: 1131-36.
14. Sitor D et al. Resistance to cefotaxime and seven other beta-lactams in members of the family Enterobacteriaceae: a 3-year survey in France. *Antimicrob. Agents Chemother* 1992; 36(8): 1677-81.
15. Sirot J. Resistance enzymatique des bacilles a Gram negatif aux cephalosporines de 3-eme generation. *Med. Mal. Infect*1989; 24-30.

REZISTENȚA LA ANTIBIOTICE BETALACTAMICE A UNOR TULPINI DE ESCHERICHIA COLI IZOLATE DIN UROCULTURI

REZUMAT

Obiective: Compararea rezistenței la betalactamine a tulpinilor de Escherichia coli (E. coli) izolate din uroculturi provenite de la pacienți din ambulator și dintr-o secție de urologie.
Material și metodă: S-au luat în studiu 526 tulpini de E. coli (350 din ambulator și 176 din secția de urologie). Germenii au fost identificați cu ajutorul galeriilor API (BioMerieux), iar testarea sensibilității la antibiotice s-a realizat prin metoda difuzimetrică Kirby-Bauer. Prelucrarea statistică a datelor, interpretarea antibiogramelor și încadrarea în fenotipuri de rezistență a tulpinilor de E. coli izolate s-a realizat cu ajutorul analizorului Osiris (Bio Rad Laboratories). Pentru evidențierea tulpinilor producătoare de betalactamaze cu spectru extins (BLSE) s-a utilizat și testul de sinergie.
Rezultate: Fenotipul sălbatic a fost reprezentat de 49,15% din tulpinile izolate în ambulator și numai de 17,04% din tulpinile provenite de la urologie.
Atât în ambulator, cât și în secția de urologie au predominat tulpinile secretoare de penicilinază, izolate într-un procent de 46,85% și, respectiv, 57,95%.
Tulpinile BLSE au predominat în secția de urologie 22,27%, în ambulator fiind izolate în procent de 0,29%.
Concluzii: S-a constatat o scădere continuă a sensibilității tulpinilor de E. coli la betalactamine, în special în secția de urologie, ceea ce atrage atenția asupra necesității monitorizării prescrierii de antibiotice.

Cuvinte cheie: Escherichia coli, uroculturi, fenotipuri de rezistență

PREVALENCE AND ANTIMICROBIAL RESISTANCE OF ACINETOBACTER BAUMANNII STRAINS ISOLATED FROM THE CARDIOLOGY DEPARTMENT

DORINA DUGAESESCU, MONICA LICKER, DELIA BERCEANU-VADUVA, MIHAELA CRACIUNESCU, LILIANA DRAGOMIRESCU, ELENA HOGEA, CARMEN TATU, DELIA MUNTEAN, MIHAELA POPA, CIPRIAN PILUT, RAMONA PIRVAN, DENIS SERBAN, ROXANA MOLDOVAN

University Of Medicine and Pharmacy "Victor Babes" Timisoara

ABSTRACT

Purpose: The study assessed the degree of microbial load of the air, surfaces, the carrier status of medical staff and patients hospitalized in the Cardiology Department of Timisoara Municipal Hospital. Research has pursued the isolation and identification of Acinetobacter baumannii strains and their behavior to antimicrobial agents.

Methods: Isolation was carried out on blood agar 5% and Mac Conkey agar, incubated at 37°C, 24h. Identification of Acinetobacter baumannii strains was based on morphological, cultural and biochemical (API 20E galleries) characters. Antibiotic susceptibility testing was done by Kirby-Bauer method and automated Vitek 2 compact system.

Results: Of the 362 samples were positive 51.65%. There were 35 strains of Acinetobacter baumannii from the total of 594 bacterial isolated strains. These came from the different surfaces (65%), aeroflora (26%) and pharyngeal swabs (9%) of 4 hospitalized patients. Concerning their phenotypic patterns, most strains of A. baumannii were included in penicilinase (PASE) producing phenotype (66%), followed by cephalosporinase producing (CASE) phenotype (20%) and 5 strains (11%) belonged to the wild phenotype. Aminoglycosides wild type phenotype was 62.8%, followed by gentamycine netylmicin (GN) phenotype (14.3%) and gentamycine G phenotype (11.4%). Only 8.6% strains were resistant to fluoroquinolones.

Conclusions: In our study A. baumannii strains don't represent a particular problem in terms of antibiotic resistance. Nosocomial potential can be represented only by the 6 multirezistente strains (beta lactams + aminogliozydes + /-quinolones). It should therefore use appropriate and effective treatment against the phenomenon of selection of microorganisms that acquire antimicrobial resistance.

Keywords: phenotype, strain, nosocomial potential

INTRODUCTION

For over 30 years, nosocomial infections caused by gram-negative bacilli were found to be a serious problem for developed, but especially for the developing countries. Among the emerging pathogens involved in nosocomial infections, Acinetobacter sp. draw attention on medical world, not as much through the wide spectrum of clinical manifestations, but especially their gravity, directly linked to the lack of effective therapeutic means against multirezistente strains (2,3,6).

MATERIAL AND METHOD

362 samples were collected during december 2008 - june 2009, from Cardiology Department in the Timisoara Municipal Hospital. Most samples were from patients (29%) and environment (aeroflora - 27% and surfaces - 22%). At a reduced rate samples were collected from the sterile water, solutions and disinfectants (13%) and medical nurses (9%).

Samples were sent to the Bacteriology Laboratory of Microbiology-Virology Department from the University of Medicine and Pharmacy "Victor Babes" Timisoara where have been processed. All the samples were inoculated on Columbia 5% sheep blood agar (bioMerieux) and selective media Mac Conkey (BioRad). Then were incubated at 37°C for 24h. Identification of Acinetobacter baumannii strains was generally based on morpho-tinctorial character (gram negative coccobacilli), cultural and biochemical tests (API 20E galleries). Sensitivity tests were done

Table I. Distribution of samples collected from the Cardiology Department

	Samples collected	Percent %
Patients		
nasal swabs	46	12.7
pharyngeal swabs	24	6.62
lung aspirates	14	3.86
urines	24	6.62
Medical staff		
nasal swabs	26	7.18
skin samples	6	1.65
Surface samples	78	21.54
Disinfectant solutions / sterile water	48	13.25
Aeroflora	96	26.51
Total	**362**	**100**

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Dorina Dugaesescu, University of Medicine and Pharmacy "Victor Babeş" Timişoara, 2A Eftimie Murgu Square, 300041, Timisoara, Romania

by disk diffusion - Kirby-Bauer method and automated Vitek 2 compact system (which allowed species identification), and it was followed by classification of phenotypic patterns (4,9).

RESULTS AND DISCUSSION

Of the total 362 samples harvested 187 were positive (51.65%). We isolated 594 bacterial strains with possible nosocomial potential, from which 35 strains (6%) were represented by Acinetobacter baumannii. These strains came from different surfaces (65%), aeroflora (26%) and 4 hospitalized patients of pharyngeal swabs (9%).

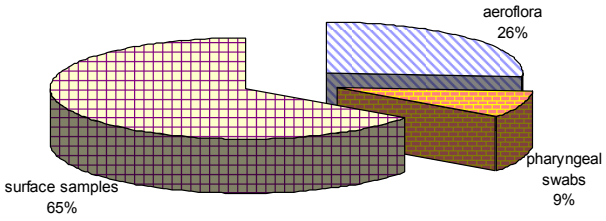


Fig. 1. Distribution of A. baumannii strains isolated from Cardiology Department

About beta-lactams resistance, most A. baumannii strains were included in PASE phenotype (66.71%), followed by CASE phenotype (20%) and 5 strains (11.42%) belonged to wild type phenotype.

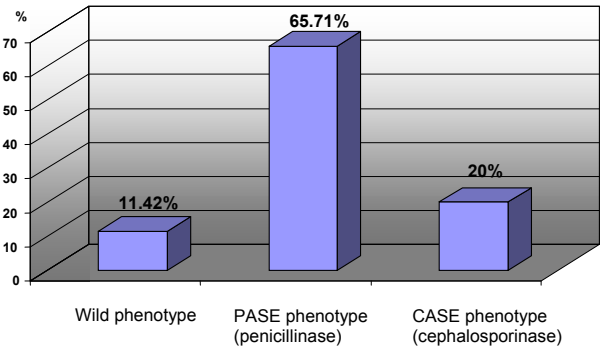


Fig. 2. A. baumannii beta-lactams resistance phenotypes

Most strains of Acinetobacter baumannii (23 isolates) were resistant to: ticarcilin, piperacillin, carbenicillin, but susceptible to cefotaxime, ceftazidime, cefoperazone, cefixime, imipenem and aztreonam. The study of aminoglycosides resistance show that, sensitive phenotype of Acinetobacter baumannii isolated strains had the largest share (62.85%), followed by GN phenotype (14.28%) and G phenotype (11.42%).

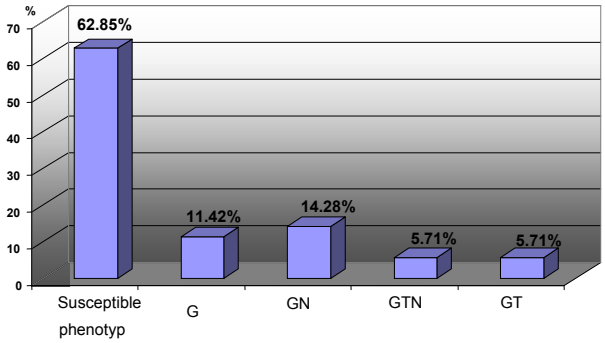


Fig. 3. A. baumannii aminoglycosides resistance phenotypes

Most strains were susceptible to fluoroquinolones (91.42%) and only (8.57%) were resistant. The same percentage was met in the case of sulphonamides (8.57%).

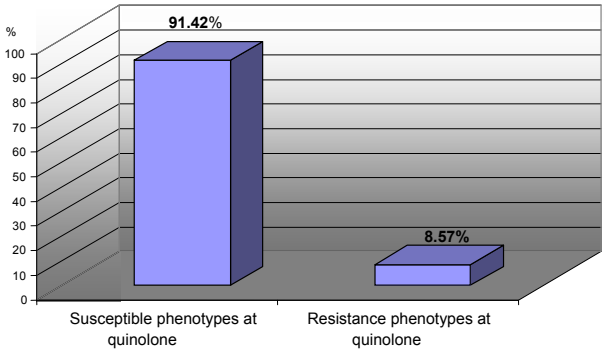


Fig. 4. A. baumannii quinolones resistance phenotypes

Entrance through a colonized patient is the most likely mode. However, introduction through contaminated materials (such as pillows) has also been documented. Notably, introduction by healthy carriers is also conceivable, although it is not known whether the rare strains that circulate in the community have epidemic potential. Once on a ward, A. baumannii can spread from the colonized patient to the environment and other susceptible patients (10). The direct environment of the patient can become contaminated by excreta, air droplets and scales of skin. Interestingly, A. baumannii can survive well in the dry environment, a feature it shares with staphylococci. Hence, the contaminated environment can become a reservoir from which the organism can spread. The acquisition of A. baumannii by susceptible patients can occur through various routes, of which the hands of hospital staff are thought to be the most common, although the precise mode of transmission is usually difficult to assess (5).

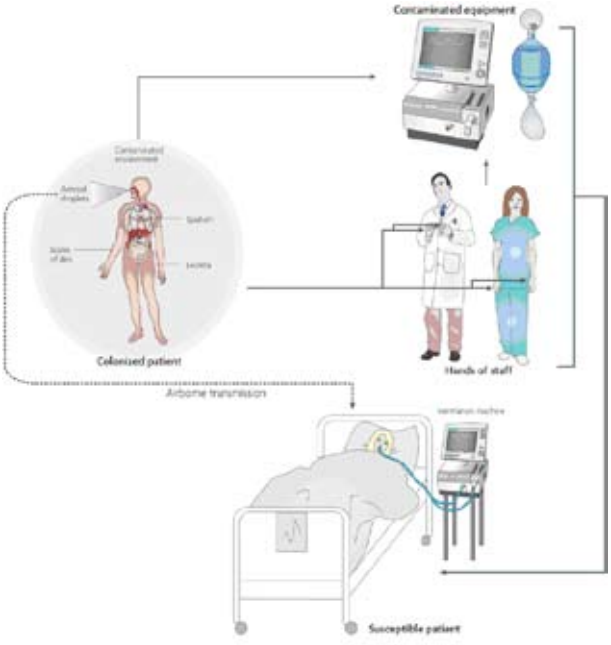


Fig. 5. The possible modes of Acinetobacter baumannii entry into a ward (5)

As shown in many literature data, Acinetobacter sp. has a significant role in colo-

nization and infection of hospitalized patients. Estimation of the real frequency of nosocomial infections produced by Acinetobacter sp. as etiologic agent is difficult, simple isolation of these microorganisms in clinical samples is not equivalent to infection. In many cases it reflects only colonization of those sites. Furthermore, clear separation between the two situations is hampered by the fact that what was originally isolated and labeled as "colonization" may, in a variable time then, clearly defined "infection". Depends on the anatomic site and time of collection, but also a complex of factors, including biological and the patient's immune status, pre-existing pathology and treatment, department's profile and effectiveness of established infection control are the most important unit. The use of molecular biology techniques in the recent years made the necessary clarifications in many cases of this kind (5,7,10).

CONCLUSIONS

1. In our study of A. baumannii strains don't represent a particular problem in terms of resistance to various classes of antibiotics. Only 6 strains were multirezistente (beta lactams + aminogliozydes + /-quinolones)
2. Good understanding of resistance phenotypes, inform us about precisely the resistance mechanisms and on the ways of spread.
3. With these data we can assess the development of resistance in the future and establish a well thought f antibiotic policy, for preventing multiplication of resistant strains.
4. The ability of these bacteria to develop antimicrobial resistance by various mechanisms, their great capacity to survive long in the hospital environment and multiple opportunities for transmission between patients, raise serious problems in the treatment and management of nosocomial infections produced by Acinetobacter sp.

REFERENCES

1. Bassetti M, Righi E, Silvano E, Petrosillo N, Nicolini L. Drug Treatment for Multidrug-resistant Acinetobacter baumannii Infections. *Future Microbiology* 2008; 3(6): 649-660.
2. Bergogne-Berezin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin Microbiol Rev.* 1996; 9:148-65.
3. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev*, 2007, 48: 933-51.
4. Branea D. Comparative Study of resistance to chemotherapy anti-microbial strains isolated from hospital and outpatient. Doctoral Thesis, 2004.
5. Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature Rewiews Microbiology* 2007; 48: 933-51.
6. Falagas ME, Karveli EA The changing global epidemiology of *Acinetobacter baumannii* infections: a development with major public health implications. *Journal Compilation European Society of Clinical Microbiology and Infectious Deaseases*, 2006; 13(2): 117-119.
7. Gordon NC, Wareham W. A review of clinical and microbiological outcomes following treatment of infections involving multidrug-resistant Acinetobacter baumannii with tigecycline. *Journal of Antimicrobial Chemotherapy* 2009; 63(4): 775-780.
8. Lortholary O, Fagon JY, Hoi AB, Slama MA, Pierre J, Giral P et al**.** Nosocomial acquisition of multiresistant *Acinetobacter baumannii*: risk factors and prognosis. *Clin Infect Dis.* 1995; 20:790-6.
9. Moldovan R, Licker M, Berceanu D, Craciunescu M, Dan L, Branea D, Hogea E. Practical laboratory support, ed. Lito UMF Timisoara, 2002.
10. Mulin B, Talon D, Viel JF, Vincent C, Leprat R, Thouverez M et al. Risk factors for nosocomial colonization with multiresistant *Acinetobacter baumannii*. *Eur J Clin Microbiol Infect Dis.* 1995; 14:569-76.

PREVALENȚA ŞI REZISTENȚA LA ANTIBIOTICE A TULPINILOR DE ACINETOBACTER BAUMANII IZOLATE ÎNTR-O SECȚIE DE CARDIOLOGIE

REZUMAT

Scop: În urma acestui studiu am apreciat gradul de încărcătură microbiană a aerului, suprafețelor şi starea de portaj a personalului medical şi pacienților spitalizați într-o Secție de Cardiologie din Timişoara. S-a urmărit izolarea şi identificarea tulpinilor de Acinetobacter baumannii din probe recoltate şi comportamentul lor la agenții antimicrobieni.
Metodă: Probele biologice au fost însămânțate pe medii de cultură: geloză sânge 5% şi Mac Conkey, după care s-au incubat la 37°C 24h. Identificarea tulpinilor de Acinetobacter baumanii s-a făcut pe baza caracterelor morfologice, culturale, biochimice (galerii API 20E). Testarea sensibilității la antibiotice s-a făcut prin metoda difuzimetrică Kirby-Bauer şi sistemul automat Vitek 2.
Rezultate: Din cele 362 probe recolate au fost pozitive 51,65%. S-au izolat 35 tulpini de Acinetobacter baumannii din totalul celor 594 tulpini bacteriene izolate. Aceste tulpini au provenit de la nivelul diferitelor suprafețe (65%), aeroflora (26%) şi din exudatele faringiene (9%) a 4 pacienți spitalizați. La beta-lactamine majoritatea tulpinilor de A. baumannii s-au încadrat în fenotipul PAZA (66%), urmat de fenotipul CAZA (20%) şi 5 tulpini (11%) au aparținut fenotipului sălbatic. La aminoglicozide fenotipul sălbatic a fost în proporție de 62,8%, urmat de fenotipul GN (gentamicină, netilmicină) (14,3%) şi fenotipul G (gentamicină) (11,4%). La fluoroquinolone doar 8,6% tulpini au fost rezistente.
Concluzii: În cadrul studiului nostru tulpinile de A. baumannii nu au pus o problemă deosebită în ceea ce priveşte rezistența la antibiotice. Potențialul nosocomial poate fi reprezentat doar prin cele 6 tulpini multirezistente (beta-lactamine+aminogliozide+/-quinolone). Se impune deci utilizarea unui tratament eficient şi adecvat împotriva fenomenului de selecție a microorganismelor care dobândesc rezistență la antibiotice.

Cuvinte cheie: fenotip, tulpină, potențial nosocomial

ANTIMICROBIAL RESISTANCE OF ESCHERICHIA COLI STRAINS ISOLATED FROM URINARY TRACT INFECTIONS

CRACIUNESCU MIHAELA, LICKER MONICA, MUNTEAN DELIA, HOGEA ELENA, BERCEANU-VADUVA DELIA, DUGAESESCU DORINA, SERBAN D, POPA MIHAELA, PILUT C, HORHAT F, TATU CARMEN, MOLDOVAN ROXANA

Victor Babes University of Medicine and Pharmacy, Timisoara

ABSTRACT

Purpose: For nosocomial urinary infections (NUI) monitoring, samples were taken between January 2008 and January 2009, from 629 patients hospitalsed in the Obstetrics and Gynecology Timisoara Hospital.

Methods: The Gram-negative Germs were identified at the specie level by biochemical testing using the API ID 32 E galleries (bioMerieux) and their antimicrobial resistance was determined by agar mini API microdilution and the Kirby-Bauer disk diffusion (on standardized subculture) methods.

Results: From the totalnumber of 207 positive urocultures were isolated some strains with high nosocomial potential and the leader were E. coli, with 130 strains. Unfortunately, only 23.84% from the E. coli isolated strains kept unaffected their natural sensibility to the antimicrobial therapy. The penicillinase producing strains represented 63.07 % and the CAZA and CHN strains represented 6.91 %. The ESBL producing strains (3.07%) associated aminoglycozides and fluoroquinolones resistance

Conclusions: The UTI, especially the NUI, become a health problem due to the increasing number of cases and the difficulty of treatment caused by the multiresistance to antimictobial treatment. The increased number of strains with acquired resistance to the antimicrobial therapy found by the present research study is underlining the importance of permanent and systematic monitoring of the phenomenon.

Key words: nosocomial urinary infections, resistance phenotypes, E. coli

INTRODUCTION

Obstetrics and gynaecology departments have always had a leading role in the history of nosocomial infections, starting with the research for the puerperal fever of Pasteur and Semmelweis (3, 7). Although huge progresses have been made regarding the microbiology, epidemiology, hygiene and the anti-infectious chemotherapy, the urinary tract infections (UTI) are still a problem of the patients hospitalized in these departments, due to the existing risk factors (1, 4).

The main risk factor, accountable for more than 80% of the nosocomial urinary infections (NUI) is the instrumental approach of the urinary tract. NUI are favoured by the probing of the urinary tract, depending on the length of time of probing, the type of drainage (the open drainage being twice more frequent incriminated than the closed drainage), the impaired integrity of the urinary tract by other gynaecological procedures or by labour complications. The intrinsic factors are: the feminine gender, the susceptibility of the host, the presence of gynaecological pathology (tumours, infections, prior antibiotic therapy), and pregnancy (2).

MATERIAL AND METHODS

Urinary samples were taken between January 2008 and January 2009, from 629 patients hospitalized in the Obstetrics and Gynaecology Timisoara Hospital. Collection of specimens was performed, generally by the hospital's medical personnel, respecting the general rules of sampling for bacteriologic analysis. The bacteriological culture media used for isolation were represented by Columbia type agar (Bio Rad, Oxoid or bioMérieux).

The samples were cultivated by calibrated loops technique on nonselective solid media agar type Columbia with 5% blood and on lactose media for enterobacteria, most frequently used being Mac Conkey (Oxoid). The germs identification was performed in concordance with their morpho-tinctorial characteristics in Gram stain, the aspect of colonies on culture media, metabolic properties and some pathogenicity tests.

The Gram-negative germs were identified at specie level by biochemical testing, the API ID 32 E galleries (bioMérieux) being used for the identification of metabolic characteristics of these germs. The antimicrobial resistance of the germs was performed by agar micro-dilutions method, using the mini API system (bioMerieux) and by the Kirby-Bauer disk diffusion method on a standardized subculture.

The extended spectrum beta-lactamase (ESBL) production in enterobacteria and other gram-negative bacilli was suspected when a reduced inhibition diameter zone surrounding the third generation of cephalosporin (cefotaxime 27 mm, ceftazidime 22 mm, ceftriaxone 25 mm), or at monobactams (aztreonam 27 mm), was noticed.

For the correct interpretation of the ESBL producing phenotype, the presence of a positive synergy test (an image like a "champagne stopper" between the cephalosporin or monobactam disks and the beta-lactamase inhibitor) is conclusive (5).

The synergy test was performed using an amoxicillin/clavulanic acid disk of in the middle, surrounded at a distance of 2 cm, by cefotaxim, ceftriaxon, ceftazidim and aztreonam disks.

RESULTS

From the total number of 207 positive urocultures, we have isolated some strains with a high nosocomial potential. These germs are represented in Figure 1. One bacterial species was identified in 205 cultures and Candida spp. was isolated in 2 samples.

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Mihaela Craciunescu, University of Medicine and Pharmacy"Victor Babeş"Timişoara, 2A Eftimie Murgu Square, 300041, Timisoara, Romania

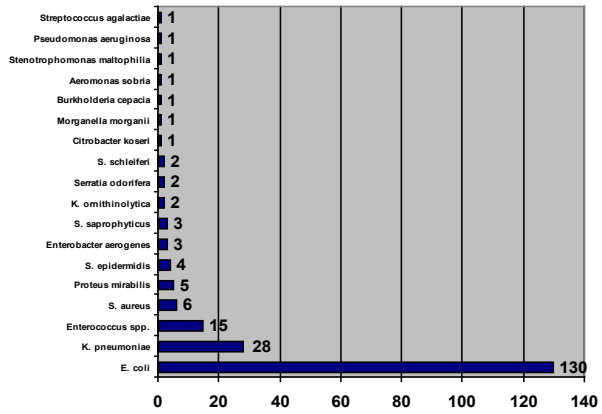


Fig. 1. Distribution of the isolated germs from the positive urocultures

As shown in the previous figure, the Gram-negative bacilli were better represented than the Gram-positive cocci, and the leader was E.coli with 130 strains.

Table I. Beta-lactams sensibility of E.coli strains

Antimicrobial chemotherapy	Sensible		Intermediary		Resistant	
	Number	Percent	Number	Percent	Number	Percent
Amoxicillin	35	26.92%	-	-	95	73.07%
Amoxiclav	84	64.61%	6	4.61%	40	30.76%
Ticarcilin	35	26.92%	-	-	95	73.07%
Piperacilin	84	64.61%	13	10%	33	25.38%
Cefalotin	35	26.92%	49	37.69%	46	35.38%
Cefuroxim	84	64.61%	-	-	46	35.38%
Cefotaxim	125	96.15%	-	-	5	3.84%
Ceftazidim	125	96.15%	-	-	5	3.84%
Ceftriaxon	125	96.15%	-	-	5	3.84%
Cefepim	126	96.92%	-	-	4	3.07%
Cefoxitin	129	99.23%	-	-	1	0.76%
Imipenem	130	100%	-	-	-	-

Table II. Aminoglycozides, quinolones, nitrofurantoin and trimethoprim-sulfamethoxazol sensibility of E.coli strains

Antimicrobial chemotherapy	Sensible		Intermediary		Resistant	
	Number	Percent	Number	Percent	Number	Percent
Gentamycin	104	80%	-	-	26	20%
Tobramycin	119	91.53%	-	-	11	8.46%
Netilmicin	123	94.61%	-	-	7	5.38%
Amikacin	124	95.38%	-	-	6	4.61%
Nalidixic acid	107	82.3%	-	-	23	17.7%
Norfloxacin	107	82.3%	-	-	23	17.7%
Pefloxacin	107	82.3%	-	-	23	17.7%
Ciprofloxacin	107	82.3%	-	-	23	17.7%
Nitrofurantoin	104	80%	-	-	26	20%
Cotrimoxazol	112	86.15%	-	-	18	13.84%

Interpretation of these results, allowed framing of the germs in phenotypic patterns.

Table III. E. coli resistance phenotypes

Associated resistance phenotypes	**Number**	**Percent**
Wild strain	31	23.84%
Low PASE	28	21.53%
High PASE	22	16.92%
CASE	6	4.61%
CHN	1	0.76%
Low PASE+IV Q	3	2.31%
Low PASE+G	1	0.76%
Low PASE+KTG+FT	3	2.31%
Low PASE+G+IV Q	2	1.53%
Low PASE+G+SXT+FT	10	7.69%
Low PASE+SXT+FT	2	1.53%
High PASE+IV Q+FT	3	2.31%
High PASE+IV Q+SXT+FT	5	3.84%
High PASE+G+IV Q+FT	2	1.53%
High PASE+KTGANt+IV Q	1	0.76%
CASE+SXT+FT	1	0.76%
CASE+IV Q	1	0.76%
KTG	1	0.76%
KTGNt	1	0.76%
KTGANt+IV Q	1	0.76%
IV Q	1	0.76%
ESBL+KTGANt+IV Q	4	3.07%
Total	**130**	**100%**

Legend: low PASE- low level of penicillinase producing phenotype, high PASE- high level penicillinase producing phenotype, CASE- inducible chromosomal cephalosporinase producing phenotype, CHN- hyperproducing cephalosporinase phenotype, ESBL- extended spectrum beta-lactamase producing phenotype, KTGANt – aminoglycozides (kanamycin, tobramycin, gentamycin, amikacin, netilmicin) resistance, IVQ- quinolones cross resistance, SXT- trimethoprim-sulfamethoxazol resistance, FT- nitrofurantoin resistance.

DISCUSSIONS

The coli bacilli are natural sensible to several families of antimicrobial drugs: beta-lactamines, aminoglycozides, fluoroquinolones, sulfamides and furans (6). Unfortunately, only 23.84% from E. coli isolated strains kept unaffected their natural sensibility to antimicrobial therapy.

The penicillinase producing strains represented 63.07% while the CAZA and CHN strains represented only 6.91%. The ELSB producing strains (3.07%) associated aminoglycozides and fluoroquinolones resistance.

We have found an increased number of strains resistant to penicillin's: aminopenicillin (73.07%) and ureidopenicillin (25.38%). 30.76% from E. coli strains were resistant to amoxicillin/clavulanic acid. We recorded a 3.07% fourth generation of cephalosporin resistant strains, 3.84% third generation cephalosporin resistance and 35.38% first and second generation of cephalosporin resistance. Resistance to cefamicin was recorded in only one strain and all the E. coli strains were sensible to carbapenem.

E.coli strains were resistant to quinolones in a 17.7% and in different percentages to aminoglycozides (between 20% for gentamycin and 4.61% for amikacin). 13.84% of the E.coli strains were resistant to trimethoprim-sulfamethoxazol, but the usage of these drugs is limited due to the contraindication during pregnancy. 20% of all the E.coli strains were nitrofurantoin resistant.

CONCLUSIONS

1. The UTI, especially the NUI, become a health problem due to the increasing number of cases and the difficulty of treatment caused by the multiresistance to antimicrobial treatment.

2. The increased number of strains with acquired antimicrobial resistance, found in the present research study is underlining the permanent and systematic necessity of monitoring of this phenomenon.

3. These prevalence data are imposing a more active participation in international surveillance studies in order to compare the results of antimicrobial resistance and to be able to implement correct methods of the infection control in our hospitals.

REFERENCES

1. Berceanu-Vaduva D. Infecţiile nosocomiale. Ed. Mirton, Timişoara, 1999
2. Bocsan IS. Practical Epidemiology for family practitioners. Ed. Medicala Universitara "Iuliu Hatieganu" Cluj Napoca, 1999
3. Holmes OW. The contagiousness of puerperal fever. *N Engl QJ Med Surg.* 1843; 1: 503-530.
4. Licker M, Moldovan R et al. Antibiotics resistence, history and actuality. *Ed. Eurostampa*, Timişoara, 2002.
5. Lorian V. Antibiotics in laboratory medicine. Lippincott Williams &Wilkins, 2005
6. Mandell GL, Bennett JE, Dolin R. Principles and Practice of Infectious Diseases. 5th ed. New York: Churchill Livingstone, 2000
7. Pasteur L. Puerperal sepsis. *Bull Acad Med* (Paris). 1879; 8: 256-260

REZISTENTA LA ANTIBIOTICE A TULPINILOR DE ESCHERICHIA COLI IZOLATE DIN INFECTII URINARE

REZUMAT

Scop: Pentru monitorizarea infecţiilor urinare nosocomiale, în perioada ianuarie 2008-ianuarie 2009, au fost prelevate probe de urină de la 629 paciente din secţiile de Obstetrică şi Ginecologie ale Spitalului Clinic „Dr. Dumitru Popescu" Timişoara.

Material si metode: Germenii au fost identificaţi utilizând galerii API (ID 32 E), iar pentru testarea sensibilităţii am folosit atât metoda microdiluţiilor în agar pe sistemul mini API (bioMerieux) cât şi metoda difuzimetrică Kirby-Bauer pe subcultură standardizată.

Rezultate: Din cele 207 uroculturi pozitive s-au izolat o serie de tulpini bacteriene aparţinând unor specii cu pronunţat potenţial nosocomial, lider fiind E.coli cu un număr de 130 tulpini. Numai 23,84% din tulpinile de E. coli pe care le-am izolat şi-au păstrat nealterată sensibilitatea naturală la chimioterapicele antiinfecţioase. Tulpinile producătoare de penicilinază au fost izolate într-un procent de 63,07%, iar fenotipurile CAZA şi respectiv CHN, într-un procent de doar 6,91%. Tulpinile secretoare de BLSE (3,07%) au asociat rezistenţă la aminoglicozide şi fluoroquinolone.

Concluzii: Infecţiile tractului urinar, în special cele cu caracter nosocomial, au devenit o adevărată problemă de sănătate prin creşterea constantă a numărului de cazuri precum şi prin dificultăţile de tratament cauzate de tulpinile multirezistente la antibiotice. Numărul ridicat de tulpini rezistente la chimioterapicele antiinfecţioase, cuprinse în prezentul studiu, subliniază încă o dată necesitatea monitorizării permanente şi sistematice a evoluţiei acestui fenomen.

Cuvinte cheie: infecţii urinare nosocomiale, fenotipuri de rezistenţă, E. coli

IN VITRO CHARACTERIZATION OF MECHANISMS INVOLVED IN ADIPOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS AND TUMOR ASSOCIATED FIBROBLASTS

FLORINA BOJIN[1], CALIN TATU[2,3], OANA GAVRILIUC[1], VALENTIN ORDODI[2,3], ALEXANDRA GRUIA[2], ADRIANA ROSCA[2], GABRIELA TANASIE[1,2], CARMEN TATU[1,2], DANIELA CRISNIC[1,2], DACIANA NISTOR[1,2], MIRABELA CRISTEA[2], SIMONA ANGHEL[1,2], ALEXANDRA BOLEMAN[4], CARMEN BUNU[1], VIRGIL PAUNESCU[1,2]

[1]Department of Physiology and Immunology, "Victor Babes" University of Medicine and Pharmacy Timisoara
[2]Department of Biology, "Victor Babes" University of Medicine and Pharmacy Timisoara
[3]Transplant Immunology Center, County Clinical Emergency Hospital Timisoara
[4]University of Agricultural Sciences and Veterinary Medicine of Banat Timisoara

ABSTRACT

The purpose of our study was to investigate the molecular mechanisms of adipogenesis regulation in different differentiation stages, both in mesenchymal stem cells (MSCs), as well in tumor associated fibroblasts (TAFs), considering their potential to transform into cells exhibiting intra-cytoplasmic lipid vacuoles. Starting with passage 2 for MSCs and passage 4 for TAFs, in appropriate culture conditions, cells were induced to differentiate towards the adipogenic lineage within 21 days. Total RNA extraction followed by RT-PCR and qRT-PCR analysis and histochemical staining were performed in certain key points of differentiation time in order to confirm adipocytic induction. Immunocytochemical staining using anti-FABP4 antibody, specific for mature adipocytes revealed in both cell types complete differentiation after 3 weeks of culture. Up- and down-regulation of different molecular markers, such as PPARγ, LPL and C/EBPα, can be juxtaposed on adipocytic development in a similar manner in both cell types studied, being a promising pathway to study accumulation of mature adipocytes and development of obesity, as well as role of adipose tissue in tumor progression.

Key words: mesenchymal stem cells, adipocytes, tumor-associated fibroblasts, adipogenesis, molecular marker

INTRODUCTION

Adipogenesis is a complex process involving proliferation of precursor cells, commitment towards the adipogenic lineage and terminal differentiation into mature adipocytes (1). Although the terminal differentiation of adipocytes is intensively studied and described using murine cell lines, key information about commitment of human precursor cells to the adipogenic lineage is still lacking.

Human pre-adipocytes derived from adipose tissue through collagenase digestion are widely used to study human adipogenesis; however, these cells are already committed to the adipogenic lineage (2). Moreover, human pre-adipocytes have reduced proliferative ability, un unpredictable variability based on different donors and anatomical sites, and limited availability (1,3). Human mesenchymal stem cells (hMSCs) (4) represent an alternative to this model, because they are multipotent precursors of several cell types, including adipocytes, osteoblasts and chondrocytes (5,6), they can be isolated from donor bone marrow and separated from other cell types by centrifugation on a density gradient. The variability between hMSCs from different donors was reported to be low, while MSCs proliferate under in vitro conditions and retain their adipogenic, chondrogenic and osteogenic potential (6). Theoretical concepts of MSCs differentiation pathway towards the adipocytic lineage can be seen in Figure 1.

Large amount of data emerged in the last years regarding the supportive role of tumor stroma, which can play a crucial role in tumorigenesis and invasion. Mechanisms used in these processes remain unknown, but different chemokines, cytokines and growth factors secreted by tumor stroma seem to induce selection and expansion of neoplastic cells (7-9). Tumor associated fibroblasts (TAFs) are considered to form the majority of tumor stroma and posses similar characteristics with MSCs in the respect of proliferation and differentiation potential (10), as well

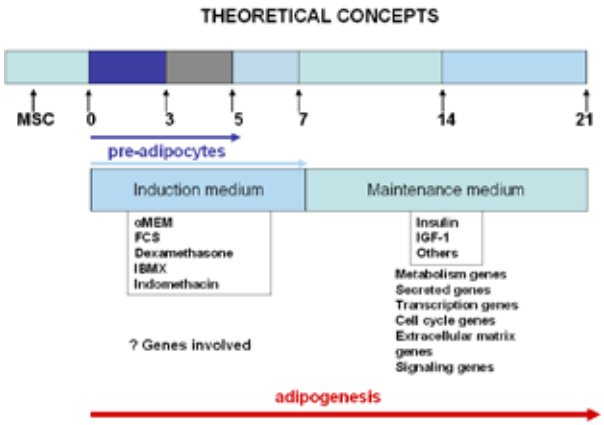


Fig. 1. Theoretical concepts of MSCs differentiation pathway towards mature adipocytes, considering the pre-adipocytes stage, medium composition and genes involved in each of these stages

Received September 2009. Accepted December 2009. Address for correspondence: Dr. Florina Bojin, Physiology and Immunology Department, "Victor Babes" University of Medicine and Pharmacy Timisoara, 2A Eftimie Murgu Square, 300041, Timisoara, Romania, e-mail: florinabojin@umft.ro

in expression of different molecular and surface markers (11,12,13). Large amounts of adipose tissue surrounds TAFs and neoplastic cells within solid tumors, so that the question rising at this point is whether TAFs are the precursors of adipocytes, and which is the role of this adipose tissue.

The main purpose of this study was to investigate the differentiation patterns of both MSCs and TAFs (isolated from breast cancer pieces) and to characterize the factors involved in adipogenic differentiation of these cell types.

MATERIAL AND METHODS

Cell isolation and culture

Normal human mesenchymal stem cells (MSCs) were obtained from bone marrow of 6 healthy Orthopedics patients undergoing hip replacement surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages and used in our experiments. The MSCs were further cultured and expanded in alpha-minimum essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37°C in 5% CO_2 atmosphere. Medium was replaced every three days and when the confluence was 80-90% the cells were passed using 0.25% Trypsin-EDTA solution (Sigma) followed by centrifugation (10 minutes, 300g) and replated in T75 culture flasks at a density of 10,000 cells/cm^2 to ensure optimal proliferation. Starting with passage two, part of the cells were used for further phenotypical analyses and differentiation assays, while MSCs expanded to passages 2-5 were used in the subsequent experiments.

Human tumor-associated fibroblasts (TAFs) were isolated using both the explant and collagenase type IV-S from Clostridium histolyticum (Sigma, St. Louis, MO, USA) methods. Breast cancer surgical pieces of approximately 5 cm^2 were obtained form 8 female patients, with the histopathological diagnosis of infiltrative ductal mammary carcinoma. Tissue-isolated cells were washed several times with phosphate buffered saline (PBS) solution, and passed through 0.70/0.40 µm strainer filters and were replated as single-cell suspension in adherent plastic culture plates.

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

Differentiation experiments were conducted at different passages for both MSCs and TAFs, starting with passage 2 for MSCs and passage 4 for TAFs (this late passage was used in order to obtain a pure cell culture). Cells were seeded in appropriate culture flasks at a cellular density of 10,000 cells/cm^2 being stimulated to differentiate in Nonhematopoietic stem cell medium for generation of adipocytes (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 1% Penicillin/Streptomycin.

Histochemical staining

Cells submitted to differentiation culture conditions were stained with Oil Red O following the manufacturer protocol in the 21st day after induction of adipocytic differentiation. Briefly, cells were washed twice with PBS, fixed in 10% solution of formaldehyde in aqueous phosphate buffer for 1 hour at room temperature, washed with 60% isopropanol, and stained with Oil Red O (Sigma) solution. Red Oil O working solution was prepared by mixing 15 ml of a stoc (0.5% in 60% isopropanol) and 10 ml of distilled water and filtering through PDVF membrane (0.22 µm) filter. Red Oil O solution stained the cells for 10 minutes, followed by repeated washing with water. Counterstaining of the nucleus used hematoxilin solution.

Immunocytochemical/immunofluorescence analysis

MSCs and TAFs differentiation experiments towards adipocytes were assessed using anti-mFABP4 as primary antibody, respectively antibody from the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems), while the visualization system was LSAB2 System-HRP (Dako); for immunofluorescence we used AlexaFluor488 and AlexaFluor546 conjugated secondary antibodies (Molecular Probes, Invitrogen) and nuclear counterstaining with DAPI (Sigma). Microscopy analysis was performed on a Nikon Eclipse E800 microscope equipped with adequate fluorescence filters.

RNA extraction, RT-PCR and qRT-PCR

Total RNA extraction was performed at different passages and in crucial day points of differentiation (day 0, 3, 5, 7, and 21) for both MSCs and TAFs, from cultured cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and RNA concentration was measured on a Nanodrop ND-1000 (Wilmington, DE, USA) spectrophotometer. For RT-PCR we used 100 ng RNA/reaction and we amplified fragments of 554 bp (PPARγ - Peroxisome Proliferator-Activated Receptor Gamma), 505 bp (LPL - LipoProtein Lipase), and 656 bp (C/EBPα – CCAAT/Enhancer Binding Protein Alpha). Total RNA (0.5 µg/reaction) from each sample was reverse transcribed to cDNA (SuperScript III First-Strand Synthesis System; Invitrogen). cDNA samples (10 ng template/ reaction) were analyzed by quantitative real-time PCR, using a LightCycler480 SYBR Green I Master system (Roche, Florence, SC, USA)) and PPARγ, LPL and C/EBPα primers. Absolute quantification was performed using the Fit Points method, with an external standard curve and molecular markers expression was reported as copy number/sample.

Gene	Sequence	Role
PPARγ	Forward: 3'-AAGACCACTCCCACTCCTTTG-5' Reverse: 3'-GTCAGCGGACTCTGGATTCA-5'	Nuclear hormone receptor, transcriptional activator, initially described as molecular target Crucial role in adipogenesis, involved in cell proliferation and differentiation in C/EBPα deficient cells Regulates development of adipocytes in response to endogenous lipid activators Low levels are present in pre-adipocytes
LPL	Forward: 3'-CTGAAGACACAGCTGAGGAC-5' Reverse: 3'-CTGGTGAATGTGTGTAAGAC-5'	Associated with differentiation process (α and β) Increases fat uptake by cells Indicator of pre-adipocytes – adipocytes conversion Marker of final stages of adipogenesis
C/EBPα	Forward: 3'-TCGACATCAGCGCCTACATC-5' Reverse: 3'-CTTGTCCACCGACTTCTTGG-5'	Decreases during adipogenesis process Positively regulates expression with PPARγ

Statistic analysis

Statistic analysis was performed using Excell Microsoft Office 2003 (Microsoft Corporation) software. The central tendencies of the variables were expressed as a mean (M), and the dispersion ones as standard deviation (sd). In order to perform the statistic comparisons, „t"-Student test and the variance analysis (ANOVA) were used for continuous variables. Differences were considered significant for $p < 0.05$.

RESULTS

Microscopy of MSCs and TAFs – derived adipocytes

MSCs are known to be able to differentiate into adipocytes in 21 days of culture in appropriate medium. The differentiation process is not involving 100% of the MSCs, but different proportions of these cells posses the characteristic of adipocytes. Spontaneous differentiation or early differentiated cells were not present in our experiments involving MSCs-derived adipocytes. After 2 weeks of culture, MSCs gained a more rounded shape, and by the end of 21 days, 50-60% of total MSCs plated were containing lipid vacuoles inside the cytoplasm, the nuclei were pushed towards the periphery and the cells began to detach from the culture flasks.

Compared to MSCs differentiation pathway, TAFs were able to differentiate even in absence of specific medium, so that in day 3 there were approximately 1-2 cells/well presenting lipid vacuoles when the cells were examined in light microscopy

with high magnification (600x). Most of the cells transformed in adipocytes by the end of 3 weeks of culture in appropriate differentiation medium, but the proportion of cells presenting lipid vacuoles was lower than in MSCs (40%). TAFs-derived adipocytes did not transform into round cells, but maintained their spindle-like shape, although the nuclei were side-located.

Histochemical staining using Oil Red O showed a better location of the lipid vacuoles within the cytoplasm, by colouring the fat droplets in red, while the counterstaining of the nuclei presented them in blue at the peripheral side of the cells (Figure 2).

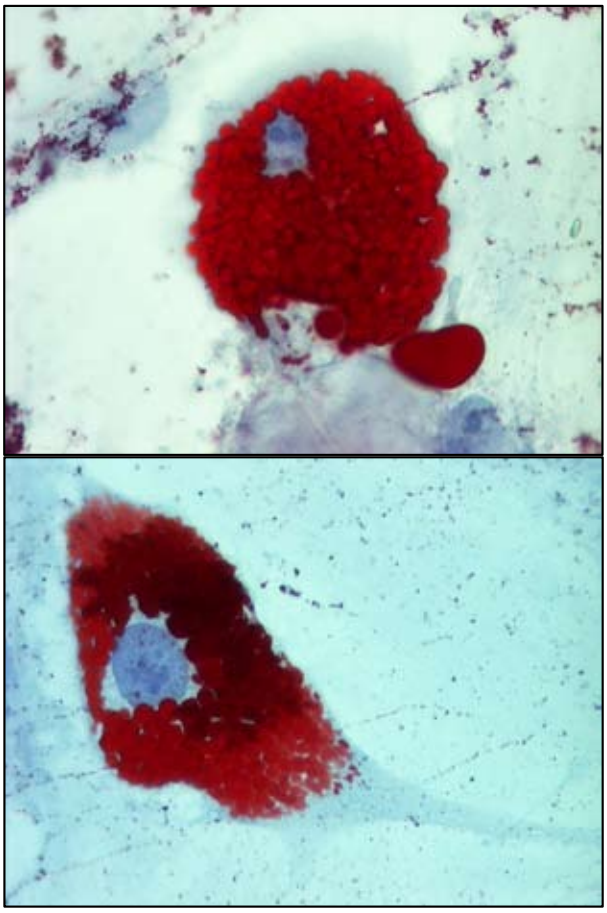
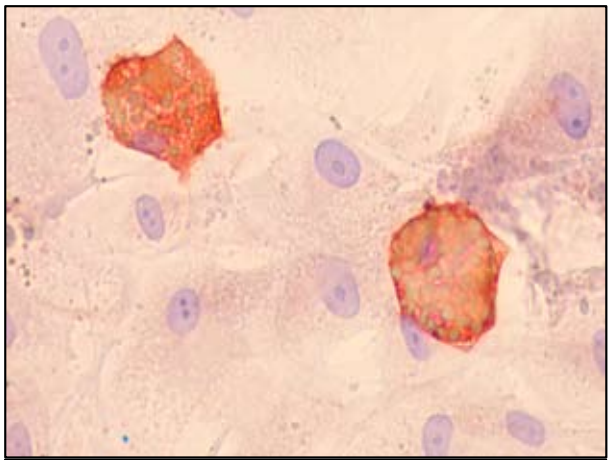


Fig. 2. Histochemical staining of MSCs (up) and TAFs (down) – derived adipocytes using Oil Red O, after 21 days of culture. Red droplets of lipids inside the cytoplasm, nuclei stained with hematoxilin (x 200)



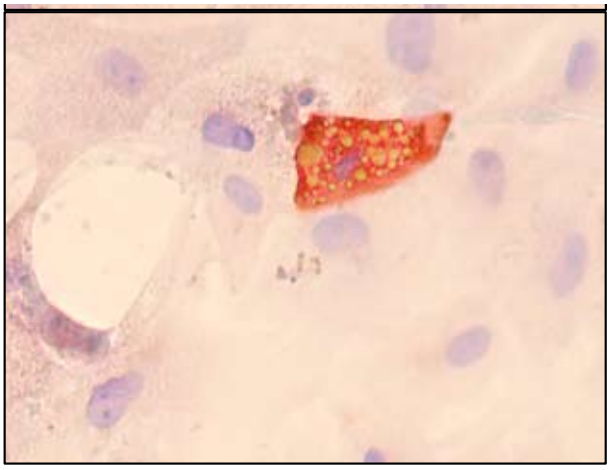


Fig. 3. Immunocytochemical staining of MSCs (up) and TAFs – derived adipocytes (FABP4) after 21 days of culture. Yellow droplets of lipids inside the cytoplasm (orange), counterstained with hematoxilin solution (x 100)

Immunocytchemical/immunofluorescence staining using anti mFABP4 antibody showed that both MSCs and TAFs were completely differentiated into adipocytes by the end of 21 days of culture, no intermediate phase presenting positive staining for this marker. We were able to count positive cells and to determine the ratio between differentiated/not-differentiated cells (Figures 3 and 4).

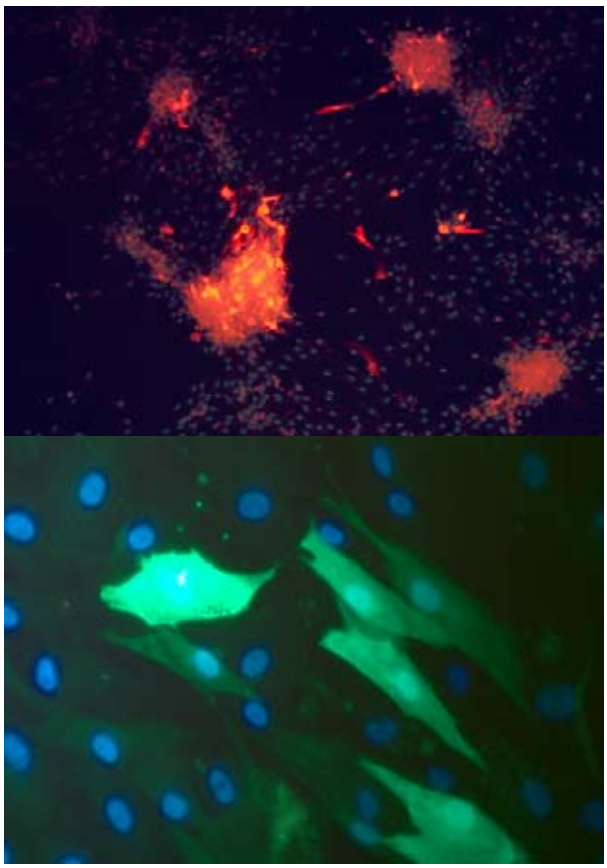


Fig. 4. Immunofluorescence MSCs (up) and TAFs (down) – derived adipocytes (FABP4) after 21 days of culture, secondary antibody conjugated with AlexaFluor488 (green fluorescence) and AlexaFluor546 (red fluorescence) (x 40)

Gene expression pattern

RT-PCR was performed on random samples of RNA for both cell types differentiated towards the adipocytic lineage in day 3, in order to determine whether they express or not the specific markers of adipocytes. All three genes were tested: PPARγ, LPL, and C/EBPα and we observed presence of amplified 554 bp, 505 bp, and 656 bp fragments respectively in all samples analyzed, even in this early phase of differentiation.

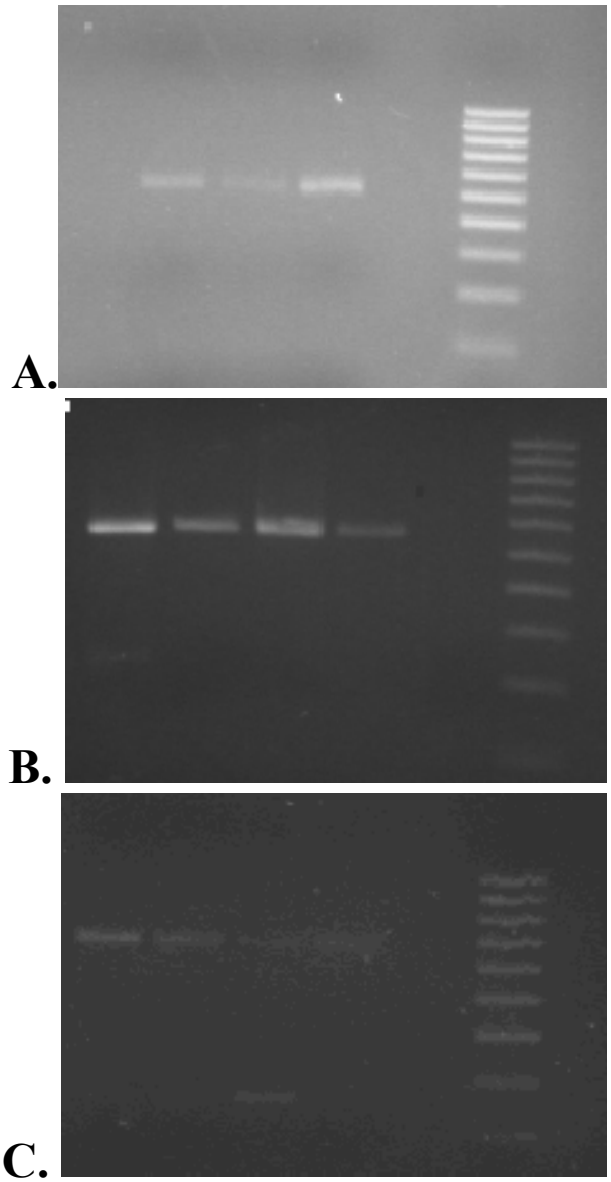


Fig. 5. RT-PCR for A. PPARγ, B. LPL, C. C/EBPα in random samples in day 3 of differentiation of both MSCs and TAFs

For quantitative analysis, we used qRT-PCR to determine relative value of genes concentration. In the beginning we wanted to test the presence of these genes in undifferentiated MSCs and TAFs, at different passages. We concluded that all 3 genes analyzed were present in both MSCs and TAFs in increased amounts at early passages, but they tend to decrease in late passages. PPARγ is down-regulated in MSCs and TAFs along with passage number, so that at passage 14 values measured were 10 times lower than at passage 4 (Figure 6). LPL was also down-regulated starting with passage 6, while at passage 14 the values were 2.5 times lower, in both cell types having almost the same value (Figure 7). While the rest of the molecular markers are down-regulated along the passage number, C/EBPα is up-regulated from passage 4 towards passage 14, when we have double amount compared with the initial one (Figure 8). This is consistent with the positive regulation of expression between PPARγ and C/EBPα described in the literature.

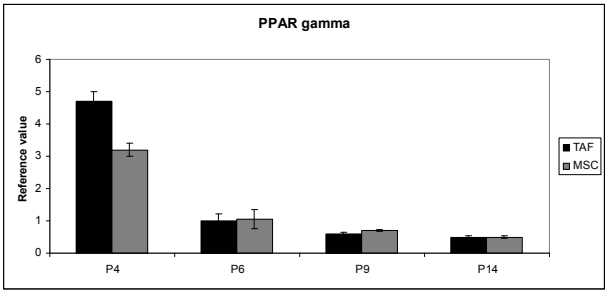


Fig. 6. PPARγ expression in MSCs and TAFs at different passages. Note the significant 10 times down-regulation of this gene in late passages of bot cell types

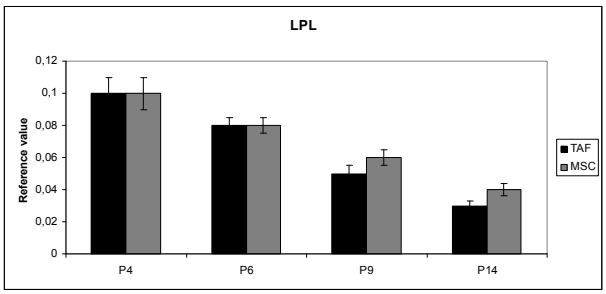


Fig. 7. LPL expression in MSCs and TAFs at different passages. Down-regulation of LPL is present in both cell types in a similar manner

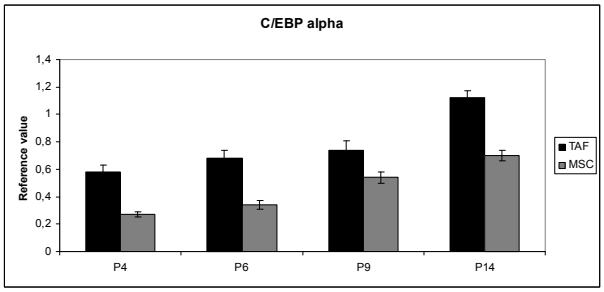


Fig. 8. C/EBPα is up-regulated progressively towards ate passages in both cell types, more significant in TAFs when compared to MSCs ($p < 0.001$)

The differentiation program was submitted to qRT-PCR analysis in key time points for both MSCs and TAFs. We analyzed the cells during the differentiation process in days 3, 5, 7 and day 21, when the experiments were stopped and we estimated the rate of differentiation process by staining techniques and presence of molecular markers. PPARγ, LPL and C/EBPα were the molecular markers used to assess the evolution of adipogenesis. Overall, all three genes presented up-regulation of their expression, starting with day 0 towards the end of differentiation pathway in day 21. PPARγ is up-regulated mainly in TAFs-derived adipocytes, while the gene maintains a relative constant value in MSCs-derived adipocytes, from day 0 towards the complete differentiation of mature adipocytes in day 21 (Figure 9). LPL, which is the gene involved in pre-adipocytes-mature adipocytes conversion, by

increasing the lipid uptake within the cells, is increasing its expression mainly after 7 days in culture, almost similar for MSCs and TAFs (Figure 10). C/EBPα maintains a low value in MSCs-derived adipocytes, but its value increases significantly (2x) in TAFs-derived adipocytes in day 21 (Figure 11). This proves that in this cells the adipogenesis process is not similar with MSCs and that we have an abnormal interrelation between the normal markers involved in generation of adipocytes.

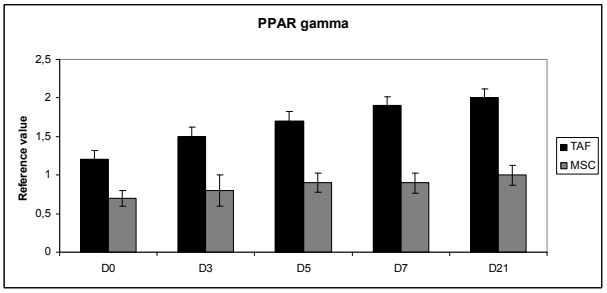


Fig. 9. Significant up-regulation of PPARγ in TAFs compared to MSCs – derived adipocytes. In MSCs the gene is maintaining a constant value ($p < 0.5$)

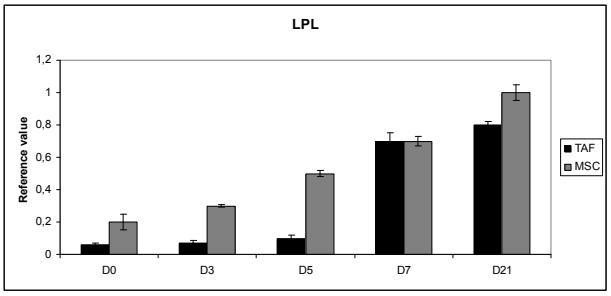


Fig. 10. Significant up-regulation of LPL in both cell types (5 x increase) proving adipocytic differentiation

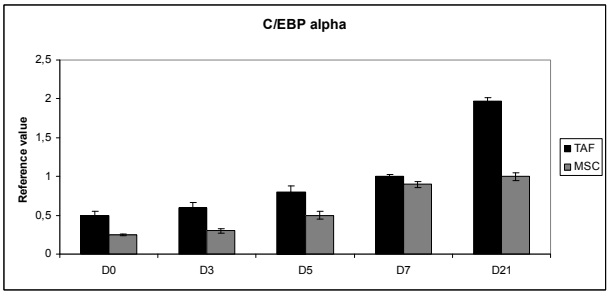


Fig. 11. Relative constant expression of C/EBPα in MSCs, while in TAFs – derived adipocytes there is a 3 x increase compared to initial values

DISCUSSION

Adipocytes develop in coordination with the vasculature, but the identity and location of white adipocyte progenitor cells in vivo are unknown (14).

A large number of cell systems were used to study adipogenic differentiation of cells from bone marrow or bone. Extensive use has been made of murine cell lines such as BMS2 (15), UAMS33 (16), and 2T3 (17), as well as line derived from p53 null mice (18). Also, human cell lines have been used: one transformed from osteoblasts (19) and other transformed from MSC (20-22). The MSCs used in the present report have the advantage that they are primary cells and they can be expanded rapidly in culture to provide adequate amounts of material to study adipogenesis in detail.

Tumor associated fibroblasts (TAFs) are recently described (11) and characterized, but their role in tumor progression and development is still unknown. They possess similar characteristics with MSCs, including surface expression markers and the ability to differentiate towards osteoblasts, chondrocytes and adipocytes. As demonstrated by our experiments, these cells can be easily differentiated and induced to transform into adipocytes, in almost the same proportion as MSCs, but the molecular marker pattern is different, suggesting an abnormal regulation of the differentiation and maturation processes. This can be a useful tool and TAFs can become an in vitro model to study adipogenesis within tumors, as well as the relation with vasculature and tumor progression.

Considering the present findings we may conclude that some molecular markers, considered characteristic for adipogenic differentiation are phenotipically present in MSCs and TAFs, being up-regulated during the differentiation process and down-regulated with passage number if no inductor is present within their environment. TAFs also exhibiting the same characteristics as MSCs could be an indicator of their common origin and development, thus contributing to accumulation of tumor-associated adipcytes. This could be a promising pathway for targeting specific molecules involved in early adipocytic differentiation, thus preventing accumulation of mature adipocytes and development of obesity, while the tumor-associated adipocytes must be further investigated for determining their role in tumor progression.

Acknowledgements

The work in this paper was supported by PNCD-II, Project 1748/2008 (financing agency CNCSIS) and Project 8844/2009 (financed by UMF "Victor Babes" Timisoara).

REFERENCES

1. Ailhaud G, Hauner H. Development of white adipose tissue. In: Bray GA, Bouchard C, James WPM, eds. *Handbook of obesity*. New York: Marcel Dekker; 1997.
2. Entenmann G, Hauner H. relationship between replication and differentiation in cultured human adipocyte precursor cells. *Am J Physiol.* 1996; 270: C10011-C1016.
3. Hauner H, Entenmann G. Regional variation of adipose differentiation in cultured stromal-vascular cells from the abdominal and femoral adipose tissue of obese women. *Int. J Obes.* 1991; 15: 121-6.
4. Pittinger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999; 284: 143-7.
5. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol.* 1999; 181: 67-73.
6. Tremain N, Korkko J, Ibberson D et al. MicroSAGE analysis of 2,353 expressed genes in a single cell derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple lineages. *Stem Cells*. 2001; 19: 408-18.
7. Liotta LA, Kohn EC. The microenvironment of the tumor-host interface. *Nature.* 2001; 411: 375-9.
8. Pupa SM, Menard S, Forti S, Tagliabue E. New insights into the role of extracellular matrix during tumor onset and progression. *J Cell Physiol.* 2002; 192: 259-67.
9. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. *Science.* 2002; 296: 1046-49.
10. Stagg J. Mesenchymal stem cells in cancer. *Stem Cell Rev.* 2008; 4: 119-24.
11. Spaeth EL, Dembinski JL, Sasser AK, et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. PLoS One. 2009; 4: e4992.
12. Singh S, Ross SR, Acena M, et al. Stroma is critical for preventing

or permitting immunological destruction of antigenic cancer cells. *J Exp Med.* 1992; 175: 139-46.
13. Sullivan NJ, Hall BM. Mesenchymal stem cells in tumor stroma. In: Bagley RG, Teicher BA, editors. Stem cells and cancer. Berlin: Springer; 2009. p. 29-38.
14. Tang W, Zeve D, Suh JM et al. White fat progenitor cells reside in the adipose vasculature. *Science* 2008 ; 322 : 583-86.
15. Kelly KA, Gimble JM. 1,25-dihydroxy vitamin D3 inhibits adipocyte differentiation and gene expression in murine bone marrow stromal cells clones and primary cultures. Endocrinology 1998; 139: 2622-28.
16. Lecka-Czernik B, Gubrij I, Moerman EJ et al. Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPAR-gamma2. *J Cell Biochem* 1999; 74: 357-71.
17. Ghosh-Choudhury N, Windle JJ, Koop BA et al. Immortalized murine osteoblasts derived from BMP 2-T-antigen expressing transgenic mice. *Endocrinology* 1996; 137: 331-9.
18. Thompson DL, Lum KD, Nygaard SC et al. The derivation and characterization of stromal cell lines from bone marrow of p53-/- mice : New insights into osteoblasts and adipocyte differentiation. *J Bone Miner Res* 1998; 13: 195-204.
19. Bodine PV, trailsmith M, Komm BS. Development and characterization of a conditionally transformed adult human osteoblastic cell line. *J Bone Miner Res* 1996; 11: 806-19.
20. Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human stromal cells. *Proc Natl Acad Sci USA* 2001; 98: 7841-45.
21. Gronthos S, Zannettino AC, Hay SJ et al. Molecular and cellular characterization of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 2003; 116: 1827-35.
22. Prabhakar U, James IE, Dodds RA et al. A novel human bone marrow stroma-derived cell line TF274 is highly osteogenic in vitro and in vivo. *Calcif Tissue Int* 1998; 63: 214-20.

CARACTERIZAREA IN VITRO A MECANISMELOR IMPLICATE IN DIFERENTIEREA ADIPOGENICA A CELULELOR STEM MEZENCHIMALE SI A FIBROBLASTELOR ASOCIATE TUMORAL

REZUMAT

Scopul acestui studiu l-a constituit investigarea mecanismelor moleculare care intervin in reglarea adipogenezei in diferite stadii de diferentiere, atat la celulele stem mezenchimale (MSC), cat si la fibroblastele associate tumoral (TAF), luand in considerare potentialul acestora de a se transforma in celule care prezinta vacuole lipidice intracitoplasmatice. Celulele aflate la pasajul 2 pentru MSC si respectiv la pasajul 4 pentru TAF au fost induse timp de 21 de zile spre linia dipocitara, in mediu de cultura specific. La anumite intervale de timp sugestive pentru diferentiere, s-a efectuat extractia ARN total, urmata de analiza RT-PCR si qRT-PCR, precum si colorarea histochimica a celulelor, pentru a confirma inucerea spre linia adipocitara. Tehnicile de imunocitochimie au utilizat anticorpul anti-FABP4, care este specific pentru adipocitele mature, relevand diferentierea completa a ambelor tipuri de celule dupa 3 saptamani de cultura. Cresterea si scaderea expresiei diferitilor markeri adipocitari, cum ar fi PPARγ, LPL si C/EBPα se poate suprapune pe modelul diferentierii adipocitare in mod asemanator in ambele tipuri de celule investigate, fiind o directie promitatoare pentru studiul acumularii adipocitelor mature si dezvoltarii obezitatii, precum si pentru determinarea rolului tesutului adipos in progresia tumorala.

Cuvinte cheie: celule stem mezenchimale, adipocite, fibroblaste associate tumoral, adipogeneza, marker molecular

NORMAL AND ABNORMAL IN MEDICINE: EPIDEMIOLOGICAL ASSESSMENT OF CHOLESTEROL LEVELS IN MEDICAL PRACTICE

BRUMBOIU IRINA[1], URSU LIGIA[2], SIMIONCA G[3], CHELARU E[4]

[1]Epidemiology Department of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca

[2]Hospital of Infectious Diseases, Cluj-Napopca, Romania

[3]1st year Intern Anatomopathology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca

[4]2nd year student at "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania

ABSTRACT

The objective of this paper was to create a model for evaluation of cholesterol levels in medical current activity. For finding the key factors for an assessment model we analyzed comparatively the levels of total cholesterol and high-density lipoprotein (HDL) in 244 patients with atherosclerotic cardiovascular diseases and in 2301 subjects without cardiovascular pathology, identified between 2003 and 2007.

In cardiovascular patient group the average value of cholesterol was 230.1 mg% and in non cardiovascular subjects, 225.2 mg%. The HDL for male patients with cardiovascular disease was agglomerated around 35-39 mg% and around 50-54 mg% for male without cardiovascular diseases. For women, the pattern was much more complex with a multimodal distribution of the cardiovascular female patients, placed in the high level area of HDL values.

In medical practice the cut-off cholesterol values are more complex to be established and more evaluations are necessaries. Usual assessment of normal and abnormal cholesterol and HDL cholesterol levels can be used for decision analysis of the necessity of a treatment or the risk of complications.

Key words: normal, abnormal values, cholesterol

INTRODUCTION

The cardiovascular diseases are the lead cause of death and disability in the developed world and in the last years became to be in the majority of in developing country. This deadly pathology has in the greatest majority of cases, the atherosclerotic nature. Lipids implication in the pathological mechanism of the atherosclerotic forming plaques was demonstrated to be very important (4, 6, 9). A better knowledge about the lipid profile in different population groups, guide to identification of some particular aspects who facilitate or represent different risks factors for cardiovascular diseases and can have implications in prophylactic and/or therapeutic recommendations (1, 3, 5, 7, 10, 12).

To understand health problems, like atherosclerotic cardiovascular diseases, the new method of modeling based on epidemiological methods, already used in medical activity for solving different problems identified in clinical activity, seems to be very useful. In the atherosclerotic pathology, for construction models, there are necessary adequate epidemiological studies for a valid selection of right parameters to be used in modeling process.

The objective of this work was to elaborate a model for the assessment of the cholesterol and HDL cholesterol levers in practical activity for caring patients with and without atherosclerotic cardiovascular diseases.

MATERIAL AND METHODS

The samples were constituted by patients with atherosclerotic cardiovascular diseases and subjects without this pathology, apparently healthy, who voluntarily participates in the period of 2003 – 2007 in our study. The including criteria were age over 18 years, both gender, persons provided from urban and rural area, voluntary participation and having some documentations signed by a medical doctor, with the registration of cholesterol and HDL cholesterol levels measured in mg% and a diagnosis. The selection intended to elevate the likelihood of the sample to be representative for regional adult population. We didn't include the patients with grave form of diseases and patients with immunodeficiency.

The tests for cholesterol levels were made in different laboratory of the north - vest region of the country, where patients included in the study were assisted for any reasons of the prophylactic or curative medical acts. Booth assistance like inpatients and outpatients were accepted. The labs of the region usually use for measurement of cholesterol levels, the enzymatic colorimetric method and for HDL cholesterol, the direct method with enzyme PEG modified and dextral sulfate.

Data registration was directly in an informatics data base, performed in Excel program of Microsoft Office Windows 2002. The confidentiality was assured by anonym registration of all data. For statistic we used Excel and EpiInfo 2002. The statistic significance was accepted at level of $p<0.05$.

Possible errors may have been induced by the method of collecting data for booth inpatients and outpatients, the utilization of all results of cholesterol levels made by different laboratories, the voluntary participation and the possibility of the duplicates because of anonym registration.

RESULTS

The sample included 2545 adult subjects, divided in two groups, first composed by 244 patients with atherosclerotic cardiovascular diseases, and the second composed by 2301 subjects without this pathology. In cardiovascular group, 63.9% were male (36.1% female) and in no cardiovascular group, male were 54.5% (45.5% female), difference statistically significant ($p<0.05$).

The cholesterol in cardiovascular patient group, had average value 230.1 mg%

Received April 2008. Accepted July 2008. Address for correspondence: Brumboiu Irina, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Epidemiology Department, 13 Emil Isac Street, Cluj-Napoca, Romania, 400023, tel. +0264-430444, +0264-594655, fax. +0264-430444, email ibrumboiu@umfcluj.ro, irinabrumboiu@yahoo.com

(IC 95%: 222.9 – 237.3), significantly higher ($p<0.05$) then 225.2 mg% (IC 95%: 222.6 – 227.9), witch was in no cardiovascular group (Table I). Cholesterol values distribution had a predomination of subjects without cardiovascular diseases in a low values area, inferior to 240 mg% and in higher values area, patients with cardiovascular diseases, became most frequently (Table I, Figure 1). The distribution of cholesterol value in female subjects with cardiovascular diseases was in plateau between 160 and 279 mg%, a very different aspect from female without that diseases, and averages values were significantly different with 225,8 mg% (IC 95%: 215.6 – 236) in cardiovascular patient group, respectively 219.3 mg% (IC 95%: 215.3 – 223.4) in female group without cardiovascular diseases (Table I, Figure 2). In male, the distribution of cholesterol levels was very similar to general aspect of all subjects included in the sample, with the predomination of cardiovascular patients in area higher then 240 mg%, and the average values of 242.2 mg% (IC 95% 232.7 – 251.7) in cardiovascular patients was significantly ($p<.05$) different from 229.7 mg% (IC 95% 226.2 – 233.2) in subjects without cardiovascular diseases (Table I, Figure 3).

Table I. Levels of cholesterol and HDL cholesterol in patients with atherosclerotic cardiovascular diseases and subjects without this pathology

Patients category	Parameter	Average value (mg %)	Standard deviation	Confidence interval (IC95%)	Variation coefficient (%)
Cardiovascular diseases, male patients	cholesterol	242,2	58,8	232.7 – 251.7	24,3
	HDL cholesterol	53.5	15.4	51.1 – 56	28.7
Cardiovascular diseases, female patients	cholesterol	225.8	47.7	215.6 – 236	21.1
	HDL cholesterol	49.9	13.9	46.9 – 52.8	28
Cardiovascular diseases, all patients	cholesterol	230.1	55.5	222.9 – 237.3	23.5
	HDL cholesterol	52.2	14.9	50.3 – 54.1	28.6
No cardiovascular diseases, male subjects	cholesterol	229.7	61.5	226.2 – 233.2	26.8
	HDL cholesterol	56.6	19	55.5 – 57.7	33.6
No cardiovascular diseases, female subjects	cholesterol	219.3	65.6	215.3 – 223.4	29.9
	HDL cholesterol	47.7	18.4	46.5 – 48.8	38.5
No cardiovascular diseases, all subjects	cholesterol	225.2	63.3	222.6 – 227.9	28.1
	HDL cholesterol	52.5	19.3	51.8 – 53.4	36.7

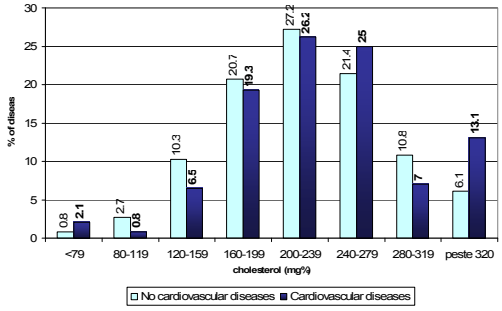


Fig.1. The distribution of patients with atherosclerotic cardiovascular diseases and subjects without cardiovascular diseases, after total cholesterol levels

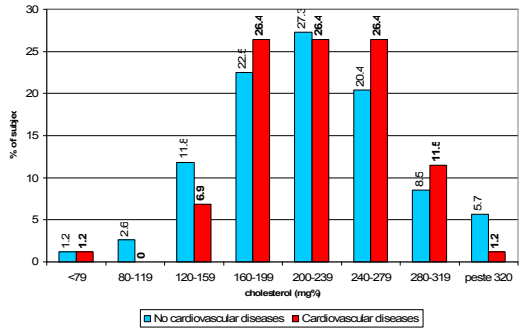


Fig.2. The distribution of female patients with atherosclerotic cardiovascular diseases and subjects without cardiovascular diseases, after total cholesterol levels

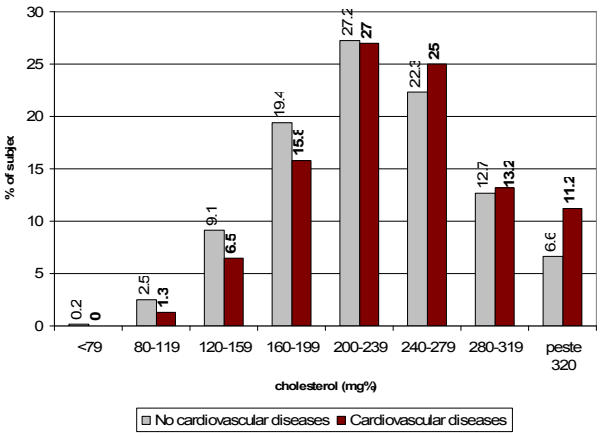


Fig.3. Distribution of male patients with atherosclerotic cardiovascular diseases and subjects without cardiovascular diseases, after total cholesterol levels

HDL cholesterol in females with cardiovascular diseases had the average value 49.9 mg% (IC 95%: 46.9 – 52.8) significantly higher then 47.7 mg% (IC 95%: 46.5 – 48.8) in females without cardiovascular diseases ($0.1<p>0.05$) (Table I). The HDL values distribution was Gaussian in female without atherosclerotic cardiovascular diseases, and with symmetric agglomeration of values around 40-44 mg% (fig. 4). Women with atherosclerotic cardiovascular diseases had a complex distribution of HDL values, with two conglomerations, one around 40-44 mg% and the second around 55-59 mg%. Men with cardiovascular diseases had a normal distribution of HDL values, with dispersion around 35-39 mg%, and thus without cardiovascular diseases was centered in 50-54 mg% (fig. 5). The average was 53.5 (IC 95%: 51.1 – 56) for cardiovascular patients and masculine gender, and 56.6 (IC 95%: 55.5 – 57.7) in men without cardiovascular diseases, difference statistically significant (Table I). The distribution of HDL for all subjects without cardiovascular diseases was relatively symmetric centered around 50-54 mg%, and for patients with atherosclerotic cardiovascular diseases, the distribution was asymmetrical with two peak around 40-44 mg% and 50-54 mg% (fig. 6). The HDL average for all cardiovascular patients was 52.2 mg% (IC95%: 50.3 – 54.1) and 52.55 mg% (IC95%: 51.8 – 53.4) for all subjects without that pathologies, no significant difference (Table I).

The average levels of total and HDL cholesterol in both cardiovascular and no cardiovascular groups for female were constantly and significantly lower then in correspondent male groups (Table I).

Variability of parameters, measured by coefficient of variation, was the highest in women without cardiovascular diseases, for total cholesterol (29.9%) and for HDL cholesterol (38.5%) (Table I). Lowers variability was in the group of women with atherosclerotic cardiovascular diseases for both total cholesterol (21.1%) and HDL cholesterol (28%) (Table I). The cardiovascular diseases groups, comparatively to no cardiovascular groups, had lowers variability for total and HDL cholesterol levels to. Constantly, HDL cholesterol had highest variability then total cholesterol in all analyzed groups.

Validity assessment of tests for total cholesterol for utilization in care assistance of cardiovascular patients was poor for cut-off at 200 mg% total cholesterol with 45.1% for sensibility and 61.7% for specificity (Table II). The sensibility of HDL cholesterol grows and the specificity low down to the highest cut-off values, and for 45 mg% cut-off, sensibility was 44.4% and specificity 55.8% (Table III).

Table II. The sensibility and specificity for different cut-of levels of total cholesterol in atherosclerotic cardiovascular diseases

Cholesterol levels (mg %)	Sensibility (%)	Specificity (%)
< 79	98	0.8
80-119	97.1	3.4
120-159	90.6	13.6
160-199	71.3	34.5
200-239	45.1	61.7
240-279	20.1	83.1
280-319	13.1	93.9
> 319	0	100

Table III. The sensibility and specificity for different cut-of levels of HDL cholesterol in atherosclerotic cardiovascular diseases

HDL cholesterol levels (mg %)	Sensibility (%)	Specificity (%)
<19	0.8	96.9
20-24	1.7	94.9
25-29	3.3	90.5
30-34	8.8	84.6
35-39	15.5	78.3
40-44	33.1	67.5
45-49	44.4	55.8
50-54	60.7	41.9
55-59	73.7	30.5
60-64	82	21.3
65-69	89.1	15
>70	100	0

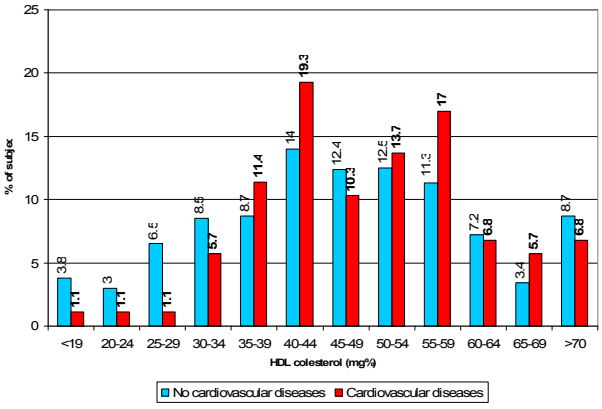


Fig.4. The distribution of female patients with atherosclerotic cardiovascular diseases and subjects without cardiovascular diseases, after HDL cholesterol levels

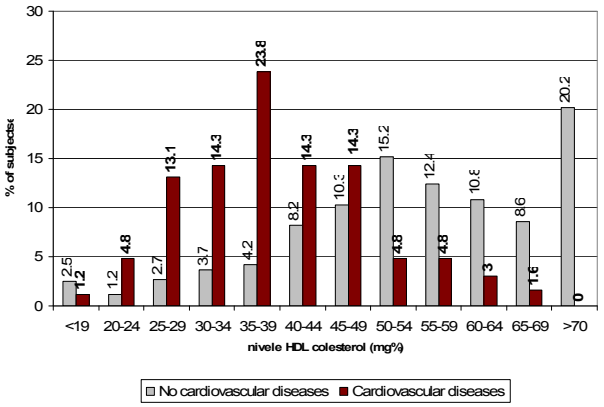


Fig.5. The distribution of male patients with atherosclerotic cardiovascular diseases and subjects without cardiovascular diseases, after HDL cholesterol levels

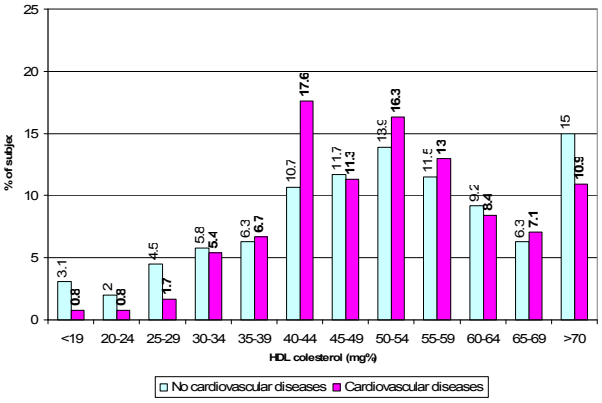


Fig. 6. The distribution of patients with atherosclerotic cardiovascular diseases and subjects without cardiovascular diseases, after HDL cholesterol levels

DISCUSSIONS

Clinical manifestations of atherosclerotic cardiovascular diseases are more frequent in male then females, who before menopause have a relatively protection conferred by estrogenic hormones who evict hypercholesterolemia (4, 8, 11). Same studies explain the differences between genders regarding cardio – vascular diseases and lipids levels, by the modification of the expression patterns and effects of genes and genes products induced by sex steroid and not by the distribution of specific alleles and genotype (8). Adder gender's differentiations are made by activities of lipoprotein lipase and hormone sensitive lipase that are sex steroid modulated. The roll of different expression induced by sex hormones became important for clinical evaluation, evolution prognostic, treatment and prevention.

Opposite to our expectations, females with atherosclerotic cardiovascular diseases had a significantly higher level of HDL cholesterol, than females without cardiovascular diseases. The explanation in part may be in regional nutritional habits with constant utilization of animal fat, usually pork and probably the modification of nutritional comportment after the disease onset (7).

High levels of serum cholesterol and low level of HDL cholesterol are known to be risks factors for atherosclerosis and because of that we considered the necessity of inclusion in elaboration of any model useful for clinical decisions (1, 11). In practice the model of cholesterols levels are insufficient for cardiovascular prediction or for other recommendation but in addition to adder factors in complex models may become useful (2).

CONCLUSIONS

In our study we observed:

- the predominance of male between the patients with atherosclerotic cardiovascular diseases;

- higher levels and a complex distribution of HDL cholesterol for women with atherosclerotic cardiovascular diseases than women without this pathology;

- for male patients, lower levels of HDL in atherosclerotic cardiovascular group;

- the levels and the variability of total cholesterol was higher for all patients with atherosclerotic cardiovascular diseases than patients without that pathology;

- constantly higher variability of HDL cholesterol than total cholesterol in all patients

REFERENCES

1. Basu AK, Pal SK, Saha S et al. Risk factor analysis in ischaemic stroke: a hospital-based study. *J Indian Med Assoc* 2005;11:586-588.
2. Brehm A, Pfeiler G, Pacini G et al. Relationship between Serum Lipoprotein Ratios and Insulin Resistance in Obesity. *Clinical Chemistry* 2004;50(12):2316-22.
3. Choi BG, Vilahur G, Yadegar D et al. The Role of High-Density Lipoprotein Cholesterol in the Prevention and Possible Treatment of Cardiovascular Diseases. *Current Molecular Medicine* 2006;6:571-587.
4. Cullen P, Rauterberg J, Lorkowski S. The pathogenesis of atherosclerosis. *Handb Exp Pharm* 2005;170:3-70.
5. Elkind MS, Sciacca R, Boden-Albala B et al. Moderate alcohol consumption reduces risk of ischemic stroke: the Northern Manhattan Study. *Stroke* 2006;1:13-19.
6. Falk E. Pathogenesis of atherosclerosis. *J Am Coll Cardiol* 2006;18:47-50.
7. Goulet J, Lapointe A, Lemieux S et al. Mediterranean Diet and Cardiovascular Disease. *Current Nutrition & Food Science* 2006;2:265-273.
8. Husslein P, Huber J, Tempfer C. Gender Specificity, Genetic Variation of Single Nucleotide Polymorphisms, and Blood Lipid Parameters. *Current Women's Health Reviews* 2006;2:87-90.
9. Kerenyi L, Mihalka L, Csiba L et al. Role of hyperlipidemia in atherosclerotic plaque formation in the internal carotid artery. *J Clin Ultrasound* 2006;6:283-288.
10. Perez-Castilon JL, Duenas-Laita A. New Approaches to Atherosclerotic Cardiovascular Disease. The Potentialities of Torcetrapib. *Recent Patents on Cardiovascular Drug Discovery* 2006;1:109-114.
11. Plesca-Manea L. *Tulburarile metabolismului lipidic*. In Editura Medicala Universitara "Iuliu Hatieganu": Patofiziologie, Plesca-Manea, Cluj Napoca, 1998, 164-188.
12. Rizzo M, Berneis K. The Role of Small, Dense Low-Density-Lipoproteins in Non-Coronary Forms of Atherosclerosis. *Vascular Disease Prevention* 2006;3:269-274.

NORMAL ŞI ANORMAL ÎN MEDICINĂ: EVALUAREA EPIDEMIOLOGICĂ A NIVELELOR DE COLESTEROL ÎN ACTIVITATEA PRACTICĂ MEDICALĂ

REZUMAT

Obiectivul acestei lucrări a fost elaborarea unui model de evaluare a nivelelor colesterolului în activitatea medicală curentă. În găsirea factorilor cheie ai unui model de evaluare am analizat comparativ nivelele colesterolului şi a high-density lipoprotein (HDL) la 244 pacienţi cu boli cardiovasculare de natură aterosclerotică şi la 2301 subiecţi fără patologie cardiovasculară, identificaţi în perioada 2003 - 2007.

Valoarea medie a colesterolului la pacienţii cu boli cardiovasculare a fost de 230,1 mg% şi de 225,2 mg% la cei fără patologie cardiovasculară. Nivelele HDL la pacienţii de gen masculin cu boli cardiovasculare s-au aglomerat în jurul valorilor de 35-39 mg%, iar în jurul valorilor de 50-54 mg% cele ale pacienţilor de gen masculin fără boli cardiovasculare. Pentru femei, modelul a fost complex, cu distribuţie multimodală a pacientelor cu boli cardiovasculare, care s-au plasat în zona nivelelor HDL cu mare risc.

În practica medicală valoarea prag pentru colesterol este dificil să fie stabilită, făcând necesară aprofundarea evaluărilor. În mod uzual, evaluarea valorilor normale şi patologice ale colesterolului şi a HDL colesterol poate fi folosită în analiza deciziei necesităţii unei atitudini terapeutice sau a riscului apariţiei unor complicaţii la boala de bază.

Cuvinte cheie: normal, valori anormale, colesterol

HARVESTING, ISOLATION AND CHARACTERIZATION OF DOG PERIOSTEUM - DERIVED CELLS

ANGHEL SIMONA[1,4], BOJIN FLORINA[1], IGNA CORNEL[5], TANASIE GABRIELA[1,4], SERES MONICA[5], TATU CARMEN [1,4], LUCA CALIN[5], TATU CALIN[2,4], ROSCA ADRIANA[4], GRUIA ALEXANDRA[4], ORDODI VALENTIN[2], GEORGESCU REMUS[3], PAUNESCU VIRGIL[1,4]

[1]Physiology and Immunology Department, University of Medicine and Pharmacy "Victor Babes" Timisoara
[2]Biology Department, University of Medicine and Pharmacy "Victor Babes" Timisoara
[3]Biophysics Department, University of Medicine and Pharmacy "Victor Babes" Timisoara
[4]Transplant Immunology Center, County Clinical Emergency Hospital Timisoara
[5]University of Agricultural Sciences and Veterinary Medicine of Banat Timisoara

ABSTRACT

The aim of the present study is to find an easy method to harvest, isolate and characterize dog periosteum-derived cells and expand these cells in vitro. In this study, periosteum from adult dogs femur was harvested and expanded.

The study evaluated and standardized the procedure for periosteum-derived cells harvesting and the optimal method for isolation. The isolated periosteum-derived cells were maintained in vitro culture condition for 60 days. In that time the cells were evaluated regarding the morphological characteristics, viability and quantification of proliferation capacity in appropriate culture conditions.

Key words: periosteum, periosteum-derived cells, isolation methods

INTRODUCTION

Bone fracture healing and bone defects involve restoration by proliferation, migration and differentiation of cells derived from the inner layers of the periosteum.

In order to study cell/biomaterial interactions, cells obtained from calvaria (1,2,3,4) and long bones (5,6) are frequently used. Disadvantages of this type of cells are related to their advanced differentiation stage. Consequently, it is difficult to expand them into large amounts or to keep them in culture for long periods (7,8). The latter renders these cells less suitable for long-term in vitro test systems. The use of progenitor cells (periosteum or bone marrow derived cells) may circumvent these problems (9, 10, 11, 12, 13).

Periosteum-derived cells are in a less differentiated stage, which may favor easier expansion and maintenance for longer periods. In the literature, several attempts to culture and differentiate periosteum- derived cells towards the osteogenic lineage were performed (14, 15, 16).

MATERIALS AND METHODS

Femoral periosteal flaps sampling

Biologic material was obtained from five common-breed dogs which were selected based on specific criteria, while data about age, gender, and weight of animals and also about types of tissue samples and sampling regions were noted, being presented in Table I.

Table I. Experimental animals' characteristics

No.	Gender	Age (years)	Weight (kg)	Periosteal flaps for digestion	Periosteal flaps for explants
C1	♂	2	21	Left and right femora	Left and right femora
C 2	♂	3	22	Left and right femora	Left and right femora
C3	♂	3	25	Left femur	Left femur
C4	♀	3	16	Left and right femora	Left femur
C 5	♂	4	19	Left femur	Left femur

Anesthetic procedure used was narco-neuroleptanalgesy (NNL-A). Premedication – 0.2 mg/kg body weight Acepromazine and 8 mg/kg body weight Ketamine. Dogs were intubated (endotracheal tube) and narcosis was induced and maintained with Halothan.

Preparation of surgical field and positioning on the surgical table

Surgical field was prepared by clipping with a hair clipper and by antisepsis. Dogs were positioned in lateral recumbence and were draped with surgical towels fixed with towel forceps.

Surgical technique – approach to the femoral shaft

The skin incision was made along the craniolateral border of the femoral bone, from the level of the great trochanter towards patella (Figure 1). The subcutaneous tissue and superficial fascia were incised directly under the skin incision.

The fascia lata was incised along the cranial border of the biceps femoris muscle aponeurosis (Figure 2). This incision extends the entire length of the skin incision.

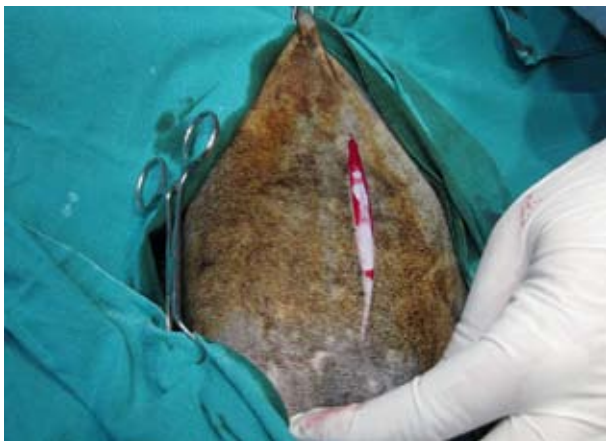


Fig. 1. Skin incision

Caudal retraction of the biceps femoris muscle and cranial retraction of the vastus lateralis muscle revealed the shaft of the femur. It was necessary to incise

Received September 2009. Accepted October 2009. Address for correspondence: Simona Anghel, 2A Eftimie Murgu Square, 300041, Timisoara, Romania, e-mail: simoanghel@yahoo.com

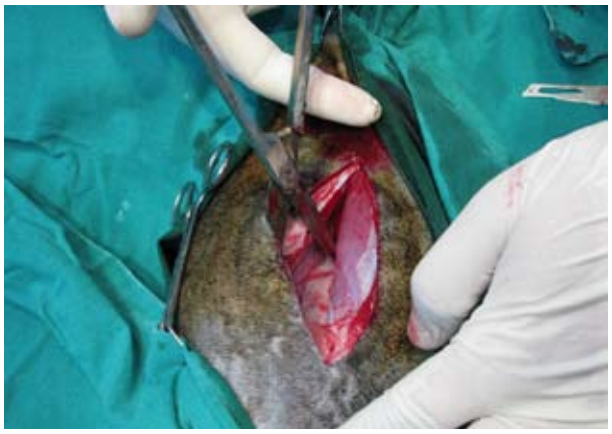


Fig. 2. Fascia lata incision

the fascial intermuscular septum between these muscles, on the lateral shaft of the bone, in order to retract adequately the vastus lateralis.

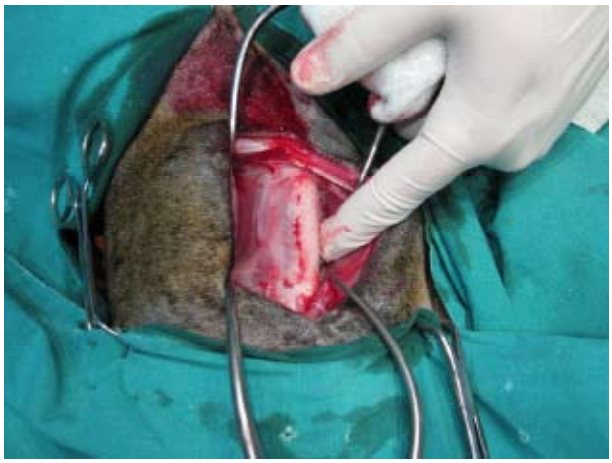


Fig. 3. Detachment of adductor muscle

Fig. 4. Detachment of vastus intermedius muscle

The adductor muscle, which inserts on the caudal aspects of the femoral shaft, was detached (Figure 3). The vastus intermedius muscle on the cranial surface of the shaft were detached by freeing the fascia between the muscle and the bone (Figure 4).

The periosteal flaps were detached using a periosteal elevator (Figure 5). Aspects of periosteal flaps can be seen in Figure 6.

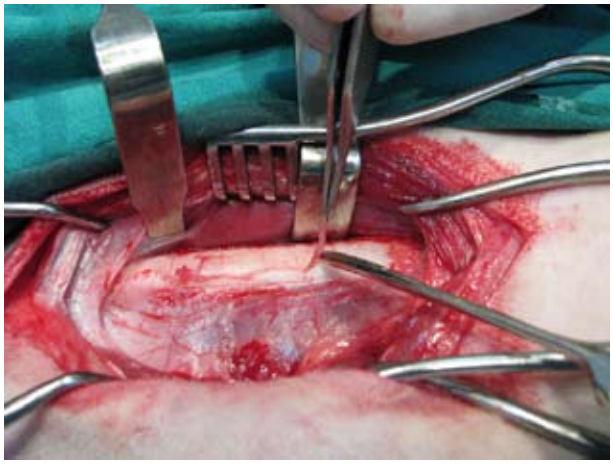


Fig. 5. Detachment of periosteal flap

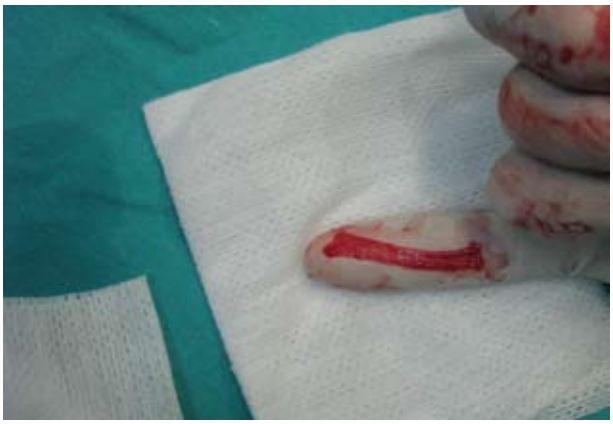


Fig. 6. Aspect of periosteal flaps

The periosteal flaps were segmented and put into:

- wells plate with osteogenic aspect superficial – Figure 7 (each wells contained approximately 1 ml of isotonic and sterile sodium chloride solution + 1% Penicillin and Streptomycin) – for explants culture;

- test tubes with the same content – for periosteal cells culture (after exposure to collagenase).

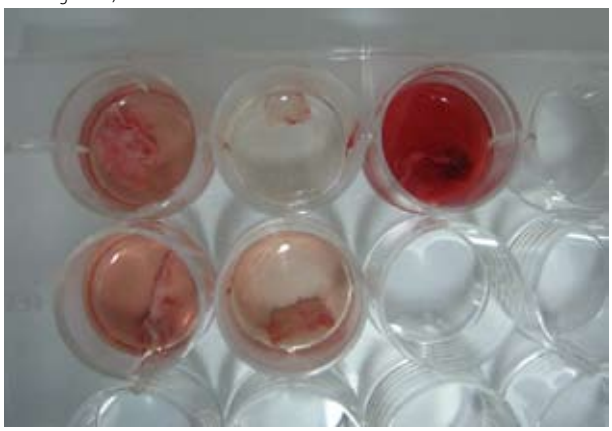


Fig. 7. Periosteal flaps in well plate

Closure of the surgical wound consisted in attaching fascia lata to the cranial border of biceps muscle by simple continuous suture with catgut, suturing the margins of the superficial facia and subcutaneous tissue in the same manner and attaching the skin by simple interrupted suture with surgical thread.

Isolation methods of periostem-derived cells

a. Digestion method

1. periosteal flaps were washed using phosphate buffer saline and were cleaned (muscle and adipose tissue remainings were carefully removed as much as possible) resulting segments were passed in sterile test tubes and covered with approximately 1 ml of type I Colagenase from Clostridium histolyticum (Figure 8).

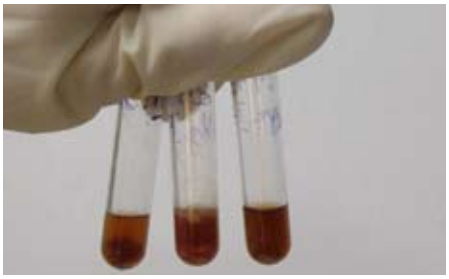


Fig. 8. Periosteal flaps aspect after adding the colagenase solution

2. test tubes containing the tissue samples were incubated at 37°C, 5% CO_2 for approximately 30-45 minutes, in order to finalize the digestion process. Time interval required for digestion was influenced by tissue sample size and the amount of fibrous tissue (Figure 9); when enzimatic digestion was finished, the resulting cellular suspensions were filtered using 70 µm strainers, passed in plastic test tubes and 20 ml of phosphate buffer saline were added to complete the first washing step;

3. diluted cellular suspensions were centrifuged at 1 500 rpm for 10 minutes (Figure 10);

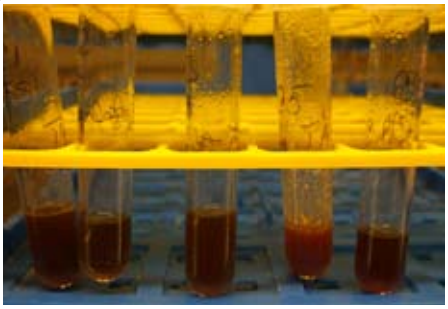


Fig. 9. Aspect of samples at the end of digestion process

4. after centrifugation, most of the supernatant was removed (approximately 19 ml). The pellet resulted due to cellular sedimentation was mechanically omogenized with the remaining supernatant, and phosphate buffer saline was added over this cellular suspension up to 20 ml (second wash);

5. cellular suspensions were centrifuged again at 1 500 rpm for 10 minutes;

6. after centrifugation, almost the entire supernatant was removed. The pellet resulted due to cellular sedimentation was mechanically omogenized with a small amount of cell culture medium (nutritive medium – HAM/F 12 + 10% fetal bovine serum + 1% Penicillin and Streptomycin, approximately 1-2 ml);

7. these cellular suspension were used for viability tests using Tripan Blue vital stainning: 10 µl of cell suspension / 90 µl of Tripan Blue, and 10 µl of this mixture were transfered into the hemacytometer for cell count and viability assessment using light microscopy. Cellular viability was above 95%. Viable cells count was determinant for choosing the size and type of culture flasks;

8. cellular suspensions were plated into culture flasks containing culture medium in variable amounts, depending on their size. Cells were plated at a cellular density of 5 x 10^4 cells/cm^2.

9. Culture flasks were then incubated into appropriate conditions: 37°C, in 5% CO_2 atmosphere.

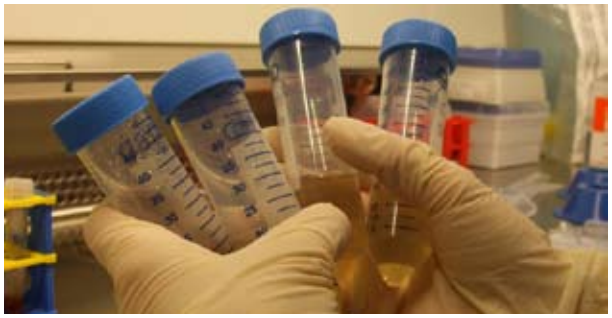


Fig. 10. Aspect of cellular suspension after the first centrifugation

b. Explant method

1. periosteal flaps were washed using phosphate buffer saline, plated with the osteogenic surface facing up, and the residual adipose and muscle tissue was removed as possible;

2. resulting segments of approximately 1 cm^2 were placed in Petri dishes with the osteogenic part in direct contact with the culture plate, and were incubated for 10 minutes at 37°C in order to provide the initial adherence;

3. adherent flaps were then covered with an appropriate amount of nutritive medium (HAM/F-12), so that to prevent floating of the periosteal segments (Figure11).

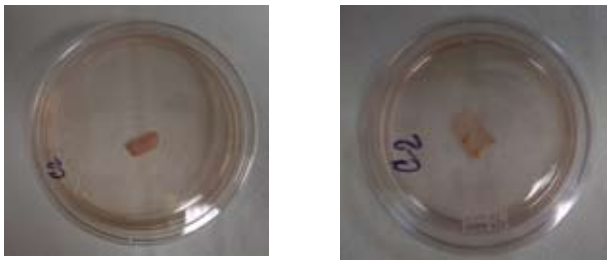


Fig. 11. Periosteal flaps adherent on Petri dshes, covered with nutrient medium

Culture of periostem-derived cells

Culture medium was replaced every 3 days, carefully analyzing the microscopic morphological changes occuring as the culture developed (Figures 12 and 13).

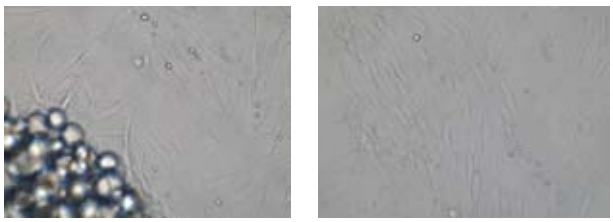


Fig. 12. Periosteum-derived cells emerging from the explant sample, Ob 20x: A. 4th day of periosteal flaps culture; B. after explant detachment

Fig. 13. Day 6 of periosteum-derived cell culture (digestion method) (Ob. 6x)

1. when reaching the confluence of approximately 90%, cells were passed using enzymatic detachment (Trysin/EDTA 0.25%), followed by neutralization

with 20% Fetal Calf Serum solution and 10 minutes centrifugation at 1500 rpm (2 successive washings). Cells were re-plated at a concentration of 5000 cells/cm^2;

2. part of the cells obtained at each passage were frozen in order to be cryopreserved and maintained for a long time. Medium used in this case was supplemented with 15% dimethylsulfoxide.

RESULTS

Periosteum surrounding the femoral bone of adult dogs (n = 5) was used. Cells from the osteogenic layers of the periost were obtained by explant cultures of periosteal fragments. Cells migrated from the tissue within 3-5 days after harvest (Figure 12). All samples gave rise to primary cell cultures with a fibroblast-like morphology, spindle-shape, and increased confluence (Figures 14, 15). In the same time interval of 3-5 days, the samples processed by digestion method induced the occurrence of fibroblast-like adherent cells within the culture flasks.

Cellular confluence (explant culture and enzymatic digestion), was reached within 10-14 days (Figures 14 and 15).

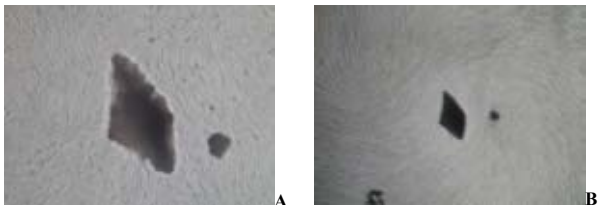


Fig.14. Explant culture confluence, ob 15x (A), ob 4x (B)

Fig. 15. Confluence of enzymatically released cells , ob 10x

Next passage cultures showed adherent cells uniformly distributed over the tissue culture dish with cells merely having a fibroblast-like morphology. Proliferation rate had a great variance depending on subjects' age and health status, proven by high periosteum-derived cell concentration / cm^2 obtained in a shorter time interval in case of young and healthy dogs. Total cell count obtained at the first passage for the explant samples varied between 1.5x10^6-2.7x10^6, depending on size of the initial sample. Compared to explant method, total cell count at first passage of samples processed by enzymatic digestion was significantly increased, variation between 3.5x10^6-7x10^6 being obtained, also depending on the size of initial periosteum sample. By in vitro expanding of the cells, at the second passage (P2) we were able to obtain from the explant samples up to 10x10^6 total cells/subject with the highest proliferation rate, while in samples processed by digestion method, total cell count was up to 15x10^6 cells/subject with the highest proliferation rate (Figure 16).

After passage 2 (P2), proliferation rate was decreasing proportionally with the number of passages and duration of cellular culture, respectively. Cells obtained using both methods were maintained in culture up to 60 days, repeatedly passed when confluence was reaching the upper limit, up to passage 5 (P5). In the last passage, cellular viability was assessed using Acridin Orange method, which established the proportion of viable cells and apoptotic cells, and allowed us to determine the

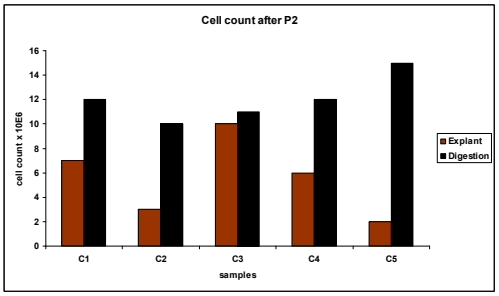
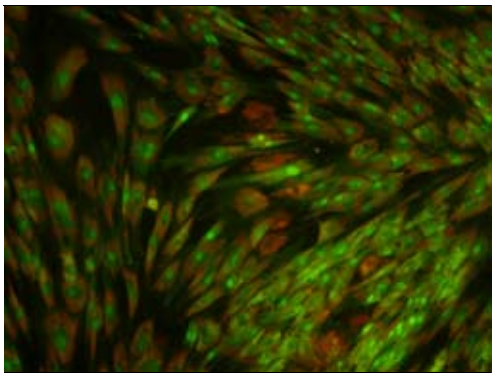


Fig. 16. Comparison between total cell count at P2 using both isolation methods, explant and enzymatic digestion

amount of cells still proliferating, which was of approximately 95% (Figure 17).

Working protocol for further cell freezing and cryopreservation proved to be optimal, considering the increased viability of cells after thawing procedures, which was maintained within 60-70%.

Fig. 17. Viability test Acridin Orange of periosteum-derived cells: viable cells-green nuclei;



apoptotic cells-orange nuclei (fluorescence microscopy, Ob. 20x)

DISCUSSION AND CONCLUSIONS

In bone fracture healing, it is important that restoration of the damaged area results in the formation of new bone, having the same integrity as the surrounding bone tissue. Cells from the osteogenic bone lining layers of the periosteum are the first contributors to the regeneration of damaged bone tissue. Therefore, it could be valuable to use these specific progenitor cells in culture models to study the osteoconductive and osteoinductive capacities of biomaterials, developed for bone regenerative therapy.

Cell cultures were obtained by migration of cells from periosteal fragments (explant culture and enzymatic digestion) and monolayer-expanded. In the literature, explant culture (14,17) as well as digestion method (16,18,19,20) of periosteal fragments are described to isolate periosteal cells, from a diversity of animals and human. However, in our opinion, explant cultures have a higher probability to result in cultures with osteogenic potential. Isolation of cells by enzymatic treatment of periosteal fragments not only liberates cells from the osteogenic lineage (deriving from the cambium layer), but also non-osteogenic fibroblasts (deriving from the fibrous layer). The periosteum present around the long bones of dogs consists of a thin layer, from which it is impossible to separate the osteogenic layer from the fibrous layer prior to enzymatic digestion. Moreover, by using periosteal tissue fragments instead of cell suspensions to start the cultures, the outgrowing cells are derived from an intact tissue (layer), a situation more similar to that in vivo when damaged bone has to be repaired. Difficulties to harvest the mesenchymal stem cells from the cambium layer are often described. This cambium layer has a tendency to remain attached to the underlying bone when the explant is taken (15). Nevertheless, even when the cambium layer together with the fibrous layer was

harvested successfully, contamination with other cell types and fibroblasts-like cells is feasible. These contaminant non-progenitor cells can inhibit the differentiation of the mesenchymal stem cells in the cultures (16,21).

Morphology was dominated by spindle-shaped, fibroblast-like cells, suggesting the contamination of the cultures with cells derived from the fibrous layer of the periosteum. The osteogenic layer was directed towards the bottom of the culture dish, allowing attachment and expansion of the cells from the cambium layer and minimizing contamination with other cell types from the upper fibrous layer. For the above mentioned reasons, we preferred working in future experiments with periosteal explants instead of enzymatically released cells.

Despite the difficulties to obtain the correct material (periosteum with an intact osteogenic layer) and despite the fact that a lot of cultures were not capable of being induced to become differentiated osteoblast cultures, periosteum-derived cultures have several advantages compared to bone derived cultures. First, there is the biological relevance of the periosteal cells being the first contributors to repair damaged bone. Secondly, their osteogenic potential persists even after prolonged cultivation in osteogenic medium. Longer culture periods (3–4 months) can be performed before final differentiation is obtained, making these cultures suitable for long-term in vitro evaluation of biomaterials (10,11). These less differentiated cells can be cultured without losing the potential of osteogenesis. As a consequence, the cells can be expanded for up to 7 passages before terminal osteoblastic differentiation. Compared to osteoblast cultures derived from long bones and calvaria from adult or fetal rat (7) which have a higher differentiation stage, periosteum-derived cultures have the advantage of prolonged cultivation before terminal differentiation and the consequent dying off of the cultures.

In conclusion, we want to emphasize the importance of periosteum-derived cell cultures to study biomaterial/cell interactions.

Acknowledgements

The work in this paper was supported by PNCDI-II Project 130/2008 (financing agency CNCSIS-Idei) and Project 41-091/2007 (financing agency CNMP).

REFERENCES

1. Attawia MA, Devin JE, Laurencin CT. Immunofluorescence and confocal laser scanning microscopy studies of osteoblast growth and phenotypic expression in three-dimensional degradable matrices. *J.Biomed. Mater.Res.*, 1995, 29: 843-48.
2. Burdick JA, Padera RF, Huang JV, Anseth KS. An investigation of the cytotoxicity and histocompatibility of in situ forming lactic acid based orthopedic biomaterials. *J. Biomed. Mater. Res. (Appl. Biomater.),* 2002, 63: 484-91.
3. Dalby MJ, Di Silvio L, Harper EJ, Bonfield W. Increasing hydroxyapatite incorporation into poly (methylmethacrylate) cement increases osteoblast adhesion and response.*Biomaterials* , 2002,23: 569-76.
4. Moursi AM, Winnard AV, Lannutti JJ, Seghi R. Enhanced osteoblast response to a polymethylmethacrylate – hydroxyapatite composite. *Biomaterials*, 2002; 23: 133-144.
5. Calandrelli L, Immirzi B, Malinconico M, Orsello G, Volpe MG, Della Ragione F, Zappia V. Biocompatibility studies on biodegradable polyester-based composites of human osteoblasts: a preliminary screening. *J. Biomed. Mater. Res.*, 2002, 59: 611-17.
6. Scotchford CA, Cascone MG, Downes S, Giusti P. Osteoblast responses to collagen–PVA bioartificial polymersin vitro: the effects of cross-linking method and collagen content. *Biomaterials,* 1998, 19: 1-11.
7. Declercq H, Van den Vreken N, De Maeyer E, Verbeeck R, Schacht E, De Ridder L , Cornelissen R. Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. *Biomaterials,* 2004, 25: 757-68.
8. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. *Macromol. Biosci.,* 2004, 4: 743-65.
9. Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yasemski MJ, Mikos AG. Bone formation by threedimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J. Biomed. Mater. Res*., 1997, 37: 17-28.
10. Perka C, Schultz O, Spitzer R, Lindenhayn K, Burmester G, Sittinger M. Segmental bone repair by tissue engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. *Biomaterials*, 2000, 21: 1145-53.
11. Arnold U, Lindenhayn K and Perka C. In vitro-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials*, 2002, 23: 2303-10.
12. Spitzer RS, Perka C, Lindenhayn K, Zippel H. Matrix engineering for osteogenic differentiation of rabbit periosteal cells using a-tricalcium phosphate particles in a three-dimensional fibrin culture. *J. Biomed. Mater.*Res. 2002, 59:690-96.
13. Lisignoli G, Zino N, Remiddi G, Piacentini A, Puggioli A, Trimarchi C, Fini M, Maraldi NM, Facchini A. Basic fibroblast growth factor enhances in vitro mineralization of rat bone marrow stromal cells grown on non-woven hyaluronic acid based polymer scaffold. *Biomaterials*, 2001, 22: 2095-105.
14. Koshihara Y, Kawamura M, Endo S, Tsutsumi C, Kodama H, Oda H, Higaki S. Establishment of human osteoblastic cells derived from periosteum in culture. *In Vitro Cell Dev. Biol*., 1989, 25(1): 37-43.
15. Breitbart AS, Grande DA, Kessler R, Ryaby JT, Fitzsimmons RJ, Grant RT. Tissue engineered bone repair of calvarial defects using cultured periosteal cells. *Plast. Reconstr. Surg*., 1998; 101: 567-76.
16. Solchaga LA, Cassie`de P, Caplan AI. Different response to osteo-inductive agents in bone-marrow and periosteum-derived cell preparations. *Acta Orthop. Scand.* 1998; 69(4): 426-32.
17. Bahrami S, Stratmann U, Wiesmann HP, Mokrys K, Bruckner P, Szuwart T. Periosteally derived osteoblast-like cells differentiate into chondrocytes in suspension culture in agarose. *Anat. Rec*., 2000, 259(2): 124-130.
18. Iwasaki M, Nakahara H, Nakase T, Kimura T, Takaoka K,Caplan AI, Ono K. Bone morphogenetic protein 2 stimulates osteogenesis but does not affect chondrogenesis in osteochondrogenic differentiation of periosteum-derived cells. *J. Bone Miner. Res*., 1994, 9(8): 1195-204.
19. De Bari C, Dell'Accio F, Luyten FP.Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis Rheum*, 2001, 44(1):85-95.
20. Gruber R, Mayer C, Bobacz K, Krauth M, Graninger W, Luyten FP, Erlacher L. Effects of cartilage-derived morphogenetic proteins and osteogenic protein-1 on osteochondrogenic differentiation of periosteum-derived cells. *Endocrinology,* 2001, 142: 2087-94.
21. Iwasaki M, Nakahara H, Nakata K, Nakase T, Kimura T, Ono K. Regulation of proliferation and osteochondrogenic differentiation of periosteum-derived cells by transforming growth factor-b and basic fibroblast growth factor. *J. Bone Joint Surg*., 1995, 77-A(4): 543-54.

RECOLTAREA, IZOLAREA SI CARACTERIZAREA CELULELOR DERIVATE DIN PERIOSTUL CANIN

REZUMAT

Scopul acestui studiu a fost identificarea unei metode simple de recoltare, izolare, caracterizare si expandare in vitro a celulelor derivate din periostul canin. In acest scop au fost folosite fragmente de periost obtinute din femurul cainilor adulti, care a fost recoltat, procesat si expandat in vitro. Studiul a evaluat si standardizat procedura de recoltare a celulelor derivate din periost si a optimizat metodele de izolare si cultivare ale acestora. Celulele derivate din periost au fost mentinute in cultura timp de 60 de zile. In acest timp celulele au fost evaluate din punct de vedere al caracteristicilor morfologice, al viabilitatii si a fost evaluata capacitatea de proliferare in conditii de cultura adecvate.

Cuvinte cheie: periost, celule derivate din periost, metode de izolare

CAVERNOUS HEMANGIOMA OF THE PAROTID GLAND

ALEXANDRA FAUR[1], ELENA LAZAR[1], MARIOARA CORNIANU[1], ALIS DEMA[1], CODRUTA LAZUREANU[1], ANCA MURESAN[1], CAMELIA GURBAN VIDITA[2], LAURA SMARANDA GOTIA[3]

[1]Department of Pathology
[2]Department of Biochemistry
[3]Department of Physiology
"Victor Babes" University of Medicine and Pharmacy Timisoara

ABSTRACT

We evaluated the medical record of patients with salivary gland neoplasms diagnosed at Timisoara City Hospital from 2002 to 2009. A study has been carried out for seven years on 220 cases of salivary gland tumors and only one case of salivary gland hemangioma was diagnosed. Although hemangioma is a common soft tissue tumor that frequently occurs in infancy, in our experience it is rarely biopsied and rather unfamiliar to the surgical pathologist. We present a case of a cavernous hemangioma involving the parotid salivary gland in a one year infant.

Key words: hemangioma, salivary gland, parotid

INTRODUCTION

Salivary glands can be divided into two distinct exocrine groups: the major salivary glands-witch includes parotid, submandibular and sublingual glands and in the mucosa of the upper aerodigestive tract there are the minor salivary glands. The major function of the salivary glands is to secrete saliva, witch plays a significant role in lubrication, digestion, immunity and overall maintenance of homeostasis within the human body (12).

Salivary gland tumors are rare disorders. The global annual incidence when all salivary gland tumors were considered varied from 0.4-13.5 cases per 100,000 populations. Hemangiomas of the salivary glands account for approximately 0.4% of salivary tumours and occur almost exclusively in the parotid gland (8). In the pediatric population hemangiomas represents about 50% of parotid neoplasms. Most infantile hemangiomas appear in the first 6 months of life and increase in size slowly and involutes by the age of 5-6 years. Hemangiomas are diagnosed usually clinically being easily noticeable, bright red, flat or raised patches on the skin, soft, warm to the touch and can swell when the baby is crying. Deeper hemangiomas may require imaging studies to confirm the diagnosis. Adult hemagioma is rare in the salivary glands (1,3,4).

Hemangiomas are benign tumors characterized by a proliferation of endothelial cells and pericytes and are seen mainly in the first two decades of life. They are classified as capillary and cavernous type. The congenital capillary type of hemangioma or the juvenile type is the predominant subtype in the first year of life. The juvenile hemangioma is common in females and there has a histologic appearance of a cellular proliferation of capillary sized vessels and pericytes that extend diffusely through the gland. The mitoses can be present. Cavernous hemangiomas are seen in older children (15).

Being a benign tumor witch eventually involutes, the treatment of hemangioma is commonly delayed. This benign vascular tumor seen in children and adults is only rarely biopsied leaving the pathologist relatively inexperienced with this clinically common lesion (1,4,6,7).

We present a case of a cavernous hemangioma involving the parotid gland of a one-year-male infant.

MATERIALS AND METHODS

We evaluated the medical record of patients with salivary gland neoplasms diagnosed at Timisoara City Hospital from 2002 to 2009. We analyzed 220 cases of salivary gland tumors and only one case of parotid hemangioma was diagnosed.

Our patient was a male infant of 1 year old witch had a large mass in the right submndibular region. Clinical examination revealed a solitary soft swelling, mobile from side to side witch due to its large size caused asymmetry of the face. The overlying skin was normal. The facial nerve was intact. This mass developed in the last four months and had approximately 5/3 cm in size. The mass was excised in toto and submitted for histopathological examination. On the cut surface it had cystic and hemorrhagic areas.

Microscopic examination on hematoxilin-eosin stain (HE) revealed a single line of endothelial cells with multiple thin-walled medium to large-sized blood vessels present in between the glandular tissue with acinar preservation (Fig.1). Many red blood cells were seen. There was no mitotic activity in the cells. The diagnosis of cavernous hemangioma was made (Fig.2).

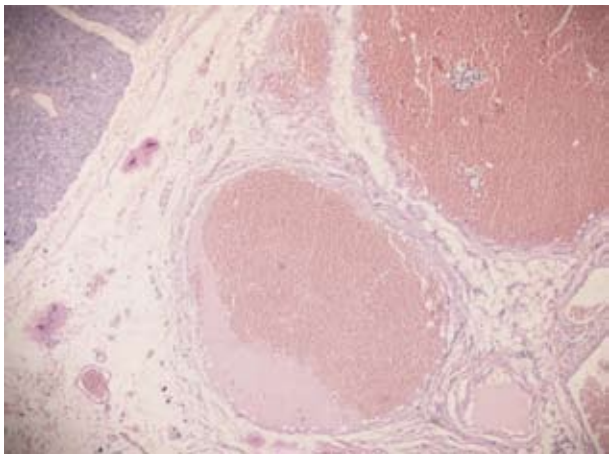


Fig. 1. Cavernous hemangioma of the parotid gland of the one-year-old male showing thin-walled vessels (right) and parotid gland parenchyma (left). HE x 4.

Received July 2009. Accepted August 2009. Address for correspondence: Dr. Alexandra Corina Faur, Department of Pathology, Victor Babes University of Medicine and Pharmacy, P-ta Eftimie Murgu 2A, 300041, Timisoara, Romania, e-mail: alexandra_pantu@yahoo.com

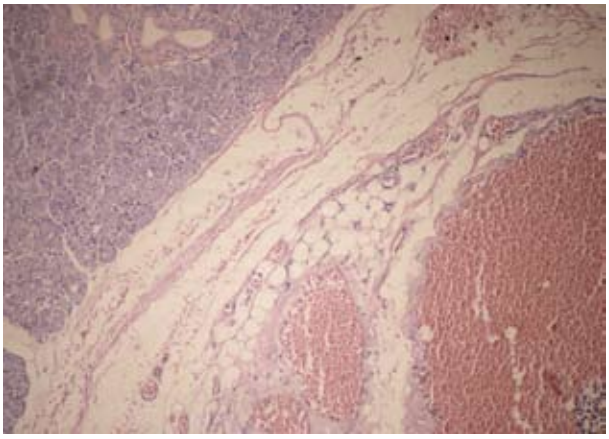


Fig.2. Higher magnification showing large diameter vessels with bland endothelial lining. HE x 20.

DISSCUSION

Neonatal and infantile lesions have a self-limiting clinical course so the treatment of infantile parotid hemangioma is usually conservative. Steroides reduce growth and are the main treatment. Pressure therapy or embolisation may be considered also (1,14,15). Surgery is performed in only few cases with increase in tumor size, disfigurement, failure to involutes or intralesional hemorrhage (9,16). In our case the tumor had a large size causing disfigurement and was localized deep in the parotid region so the surgical treatment was performed.

The differential diagnosis for hemangioma includes lymphangioma, pleomorphic adenoma, branchial cleft cyst, lymphoepithelial lesions, reactive adenopathy, abcess and cellulitis. The inflammatory lesions respond to antibiotics. The masses without signs of inflammation can be cystic or solid. Cystic lesions are commonly hemangiomas and lymphangiomas. For the lesions with a solid character magnetic resonance imaging, computer tomography is useful. (1,2)

In a study over a period of 30 years, Childers E LB et al identified only 10 cases of hemangioma located in salivary glands: nine in the parotid gland and one in a minor salivary gland in the lower lip (4). Bentz BG et all reported 324 masses of the salivary gland region in children of witch 192 were hemangiomas, followed by lymphangiomas and pleomorphic adenomas (2). The most common neoplasm found in a large study of 420 pediatric salivary tumors made by AFIP was pleomorphic adenoma followed by hemangiomas (10). Mayo Clinic rewied a 52-years experience of 74 pediatric salivary gland tumors and the most common lesion was pleomorphic adenoma followed by hemangioma also.(5)

CONCLUSION

The incidence of hemangioma cases is variable. Because the surgery is performed only on hemangiomas demonstrating an increase in tumor size, rapid tumor growth, disfigurement, failure to involutes or intralesional hemorrhage the true incidence of this lesion might be underestimated.

In conclusion although hemangioma is considered one of the most common soft tissue tumors, in our surgical pathology files is very rare and therefore is often unfamiliar to the surgical pathologist. The rarity of salivary gland hemangioma in our experience prompted us to report this case.

Acknowledgements: This work was supported partially by grant for scientific research from CNCSIS-PNII-Resurse Umane, type: TD, code CNCSIS: 314, contract no. 138/15.09.08

REFERENCES

1. Barnes L, Eveson JW, Reichart P et al. Tumours of the Salivary Glands.World Health Organization Classification of Tumours. Pathology & Genetics. *Head and Neck Tumours*. 2005;5: 209-281.
2. Bentz BG, Huges CA, Ludemann JP, et al. Masses of the Salivary gland region in children. *Arch Otolaryngol Head Neck Surg* 2000;126:1435-1439.
3. Cheuk W, Chan JKC. Salivary gland tumors. In: Christopher D. M. Fletcher's Diagnostic Histopathology of tumors, Vol.I. 2007;7:239-325.
4. Childers E LB, Furlong MA, Fanburg-Smith JC. Hemangioma of the Salivary Gland: A study of ten cases of a rarely biopsied/excised lesion. *Ann of Diagn Path* 2002;2;339-344.
5. Chong GC, Beahrs OH, Chen MLC et al. Management of parotid gland tumors in infants and children. *Mayo Clin Proc.*1975;50:279-283.
6. Dinehart SM, Kincannon J, Geronemus R: Hemangiomas: Evaluation and treatment. *Dermatolog Surg* 2001;27:475-485.
7. Drolet BA, Esterly NB, Friedl WG: Hemanmgiomas in children. N Engl J Med 1999;341:173-181
8. Ellis GL, Auclair PL. Tumours of the salivary glands. 3rd ed. Armed Forces Institute of Pathology, 1996, Washington.
9. Esposito C, Zupi A, Califano L. Surgical therapy of parotid hemangiomas. *Pediatr Surg Int* 2001, 17:335-337.
10. Krolls SO, Trodahl JN, Boyers RC. Salivary gland lesion in children: a survey of 430 cases. *Cancer*, 1972;30:459-469.
11. Maaita JK. Oral tumors in children: a review. *J Clin Pediatr Dent* 24: 134-137.
12. Myers E, Ferris R. Salivary gland disorders, 2007, Springer-Verlag, Berlin Hidelberg.
13. Tröbs RB, Mader E, Friedrich et al. Oral tumors and tumor-like lesions in infants and children. *Pediatr Surg Int* 2003,19:639-645.
14. Tryfonas GI, Tsikopoulos G, Liasidou et al. Conservative treatment of hemangiomas in infancy and childhood with interferon-alpha 2a. *Pediatr Surg Int* 1998, 13:590-5934.
15. Weiss SW, Goldblum JR. Enzinger and Weiss's Soft Tissue Tumors. Washington, Mosby, St.Louis, 4th ed., 2001
16. Werner JA, Dünne AA, Folz BJ et al. Current concepts in the classification, diagnosis and treatment of hemangiomas and vascular malformations of the head and neck. *Eur Arch Otolaryngol* 2001, 258:141-149.

HEMANGIOM CAVERNOS DE GLANDA PAROTIDA

REZUMAT

In studiul nostru, am evaluat cazurile existente in arhiva Spitalului Clinic Municipal de Urgenta Timisoara si raportate in perioada 2002-2009, selectand pacientii cu diagnostic de tumori ale glandelor salivare. Astfel ca, pe o perioada de 7 ani, din cele 220 de tumori ale glandelor salivare raportate doar un singur caz a fost diagnosticat cu hemangiom salivar cu localizare parotidiana. Desi hemangioamele sunt tumori de tesuturi moi frecvent diagnosticate in perioada copilariei in experienta nostra s-au dovedit a fi foarte rar biopsiate ceea ce le face sa fie putin cunoscute de catre patolog. In lucrarea de fata prezentam un caz de hemangiom cavernos de glanda salivara parotida la un copil de 1 an.

Cuvinte cheie: hemangiom, glanda salivara, parotida

CLINICAL ASPECTS OF AORTIC COARCTATION IN SMALL CHILDREN

ANA LASCU[1], CAIUS G. STREIAN[1], ION SOCOTEANU[1], CORINA VERNIC[2]

[1]University of Medicine and Pharmacy "Victor Babes" Timisoara, Department of Heart Surgery, Institute of Heart Disease Timisoara, Romania

[2]Departament of Medical Informatics and Biostatistics, "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania

ABSTRACT

The aortic coarctation is a relatively frequent congenital cardiac malformation consisting in the narrowing of the aortic isthmus. We compared the demographic (age, sex, environment) and medical (blood pressure, heart rate, femoral pulse) data between a group of 13 patients with aortic coarctation (group B) and a control group of 22 children (group A) with similar background and age (1-3 year).

Using the paired t test there were no important differences between left and right arm systolic blood pressure in group A ($p>0.05$). For group B there were important differences between left and right sistolic blood pressure in females ($p=0.03840$ S) but not in males. Also, the absence of the femural pulse was significantly more frequent in group B compared to group A ($p<0.0001$ES). Associated cardiac lesions as bicuspid aortic valve were found more frequently in group B (38.46%) as in group A (9.09%).

In conclusions, clinical signs of disease like the absence of femoral pulse and hypertension are specific for the presence of aortic coarctation. Furthermore, other cardiac malformations, like bicuspid aortic valve are often associated with aortic coarcation.

Key words: aortic coarctation, blood pressure, heart rate, femoral pulse.

INTRODUCTION

Aortic coarctation is a congenital malformation consisting in the narrowing in the aortic isthmus, near the origin of the left subclavian artery. It is caused by anomalies in the development of the 4th and 6th aortic arches. The aortic coarctation is relatively frequent (5-10% of all congenital cardiac malformations), with an occurrence of 5/10000 of live newborns and a gender ratio M:F of ~2:1 (1). The age of diagnosis is related with the severity of the obstruction, the clinical severity and the correct interpretation of the symptoms: a complicating factor is that aortic coarctation may be associated with other cardiac lesions. The preferred treatment is interventional or surgical, but there is the chance of relapse (3, 4). The life expectancy is low, 20% live to reach 50 of age (2, 5).

The purpose of our study is to improve the diagnosis of the aortic coarctation in order to render the proper treatment.

MATERIAL AND METHOD

In a retrospective study, there were evaluated the demographic (age, sex, environment) and medical (blood pressure, heart rate) data. The statistical analysis consisted in a quantitative and qualitative study of the data. For the quantitative analysis the data defined as an average of standard deviation; and for the qualitative analysis the data is presented as frequency.

To compare the means, we used the paired and unpaired t test and to compare the percentages we used the chi square test (χ^2). The value $p<0.05$ was considered statistically significant. The statistical analysis was done on specialized software OpenEpi 2.3 and Microsoft Excel.

The patients were divided in two groups that had similar background and age: group A (control) comprised 22 children; group B comprised 13 patients with aortic coarctation diagnosed after birth at the Dept. of Heart Surgery of The Institute of Heart Disease Timisoara in between 1998-2008. The children in both groups were in between 1-3 years old. The appraisal of the data was necessary for the comparative study and to underline the clinical and diagnostic particularities of each group.

The group A of the study was formed by 22 subjects (9 girls representing 40.91%, 13 boys representing 59.09%) with a 1.86 ± 1.05 year average (1-3 years), 14 patients for rural environment (representing 63.64%), respectively 8 patients urban environment (representing 36.36%). The B group was represented by 13 patients of which 3 girls (23.08) and 10 boys (76.92%), with an average of 2.07 ± 1.24 years, 6 patients from urban environment (46.15%) and 7 patients from rural environment (53.85%). Regarding gender distribution and origin, there were no significant differences (chi square test, $p>0.05$) between the control grup (group A) and B (13 patients with aortic coarctation) (Figures 1 and 2).

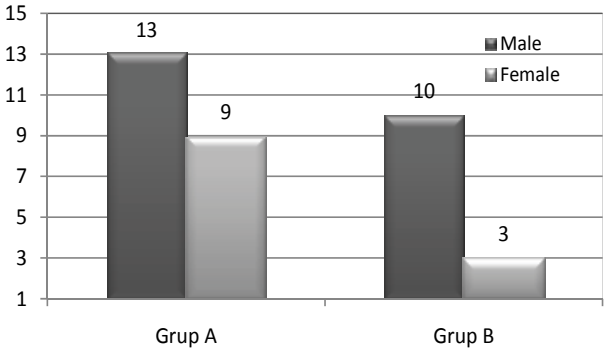


Fig. 1. Gender distribution of the tow groups

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Ana Lascu, University of Medicine and Pharmacy "Victor Babeş" Timişoara, 2A Eftimie Murgu Square, 300041, Timisoara, Romania

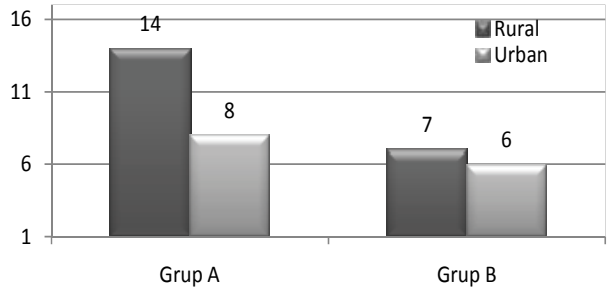


Fig. 2. Distribution according to the background

We included systolic blood pressure (SBP) and diastolic blood pressure (DBP) on the left arm respectively right arm, and heart frequency (HR) values as clinical data to evaluate the group. Also, the change in femoral pulse (weak/ absent) for the children with CoAo was compared with that of the control group.

The blood pressure measurement was made with a sphygmomenometer, using the ascultatory method (Korotkow) at the upper and lower level of the listening with a stethoscope placed at the level of humeral (brachial) artery. The noise appears at the slow decompression of the cuff, due to turbulent blood pressure.

Measurements were made in clinostatism using different sized cuffs eccording to the patients size. The width of the cuff must be at least 40% of the arm circumference (measured at the half distance between the olecron and the acronion).

The heart rate measurement was carried out by listening the sounds of the heart with a stethoscope placed on the thorax. Examining the absence or presence of the femoral pulse was performed by palpation of the femoral artery at the groin.

RESULTS AND DISCUSIONS

Using the paired t test to compare the left sistolic blood pressure with the right sistolic blood pressure in females, respectively in males from the A group, there were found no significant differences (p>0.05).

For the B group, there were no significant differences (p>0.05) in males, but there were significant differences in females, comparing SBPleft-SBPright (p=0.03840 S) and DBPleft-DBPright (p=0.01378), according to Table I. Heart rate compared between males and females was highly significant p=0.003138 in group A and p<0.001 ES in group B (Table II).

Table I. Comparative aspects of blood presure values

Measured values	Parameters	SBP_{left}(mmHg)		SBP_{right}(mmHg)		DBP_{left}(mmHg)		DBP_{right}(mmHg)	
		M	F	M	F	M	F	M	F
Groups A (22)	Ratio	80.18	79.67	80.18	83.60	49.88	52.85	52.77	54.66
	Standard deviation	20.35	29.25	14.04	15.87	17.99	26.28	10.57	16.44
	Minimum	45	51.5	30	36.5	42.5	40	30	30
	Maximum	119	115	80.5	75	118.5	107.5	67.5	72.5
GroupsB (13)	Ratio	116.56	120	107.56	110	67	73.33	60.68	66.66
	Standard deviation	17.69	15.27	18.94	8.66	10.13	2.88	7.68	11.54
	Minimum	90	100	80	105	65	70	52.5	60
	Maximum	137.5	130	127.5	120	80	75	72.5	80
p (unpaired t test)		p<0.001 ES		p<0.001 ES		0.003573 FS	0.088 4 FS	0.024 8 S	0.02725 S

Also, the change in femoral pulse (weak/ absent) was highlighted for the children group with CoAo (group B) compared with the control group (p<0.0001ES). For the group A the femoral pulse was present in 20 subjects (90.90%) and absent in only 2 subjects (9.09%), compared with group B where the femoral pulse was present in only one patient (7.69%), for the rest of 12 (92.30%) being absent.

Measured values	Parameter	HR	
		M	F
Groups A (22)	Ratio	110	125
	Standard deviation	12.85	14.45
	Minimum	105	110
	Maximum	140	155
Groups B (13)	Ratio	114	137
	Standard deviation	13.72	15.27
	Minimum	100	120
	Maximum	130	150
p (unpaired t test)		P>0.05N S	P=0.02 636 S

Table II. Comparative aspects of heart frequency values.

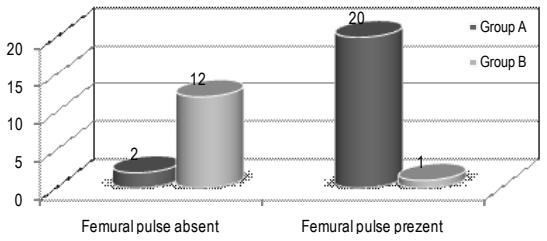


Fig.3. Modification of the femural pulse in Group B vs. Group A

The aortic coarctation diagnosis was the first to be taken into account for 8 of the children with aortic coarctation (61.58%) compared with the 22 subjects of group A (62.85%) for which the aortic coarctation was not primarly diagnosed; they were examined within a routine consultation or for other pathology by the family doctor, p>0.05 NS. The weight of the associated diagnoses is also similar, the most frequent being the bicuspid aortic valve (6 cases for group B (46.15%), followed by aortic stenosis.

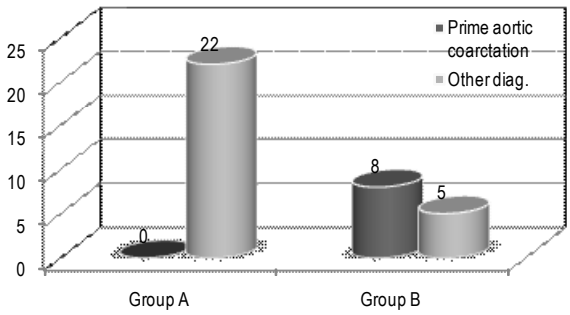


Fig.4. Primary diagnosis of aortic coarctation in Group B vs. Group A

CONCLUSIONS

Patients with aortic coarctation (aged 1-3 yrs old) represent a significant percentage (37.14%) of the total number of children aged 1-3 year old integrated in this study.

As associated diagnoses, bicuspid aortic valve is discovered most often in the group with aortic coarcation 5 patients (38.46%) as compared with the control group were bicuspid aortic valve was shown in only 2 cases (9.09%), p=0.9657 NS.

Statistical results indicate that clinical signs of disease (the absence of femoral pulse) and hypertension were evident for the children with aortic coarctation (1-3 years) as compare to group A.

REFERENCES

1. McBride KL, Marengo L, Canfield M, Langlois P, Fixler D, Belmont JW. Epidemiology of Noncomplex Left Ventricular Outflow Tract Obstruction Malformations (Aortic Valve Stenosis, Coarctation of the Aorta, Hypoplastic Left Heart Syndrome) in Texas, 1999 –2001, BirthDefects. *Res A Clin Mol Teratol*, 2005 August; 73(8): 555-561.
2. Campbell M. Natural history of coarctation of the aorta. *Br. Heart J.* 1970 September; 32 (5): 633-640.
3. Socoteanu. *Tratat de Patologie Chirurgicală Cardiovasculară,* Editura Medicală Bucureşti, 2007, Vol I, p.462-496.
4. Socoteanu. Tratat de Cardiopatii Congenitale, Editura Academiei-Bucureşti 2009, în curs de apariţie.
5. Verheugt CL, Uiterwaal CS, Grobbee DE, Mulder BJ. Long-term prognosis of congenital heart defects: A systematic review. *Int J Cardiol*, 2008 August; 131(1): 25-32.

ASPECTE CLINICE SUGESTIVE PENTRU COARCTAȚIA AORTICĂ LA COPILUL MIC

REZUMAT

Coarctația de aortă (CoAo) este o malformație cardiacă congenitală relativ frecventă constând în îngustarea lumenului aortei istmice. Au fost comparate date demografice (vârsta, sex, mediu) şi medicale (tensiunea arterială, frecvența cardiacă, pulsul femural) la un grup de 13 pacienți cu coarctație de aortă (grupul B) şi un grup de control de 22 de copii (grupul A) proveniți dintr-un studiu populațional asemănător din punct de vedere al mediului de proveniență şi al vârstei (1-3 ani).

Aplicând testul t pereche diferențele sunt nesemnificative între tensiunea arterială sistolică măsurată la brațul drept şi stâng în grupul A (p>0,05). În grupul B, diferențele sunt semnificative între tensiunea arterială sistolică dreaptă şi stângă la femei (p=0,03840 S), dar nu la sexul masculin. De asemenea, absența pulsului femural este semnificativ evidentă în grupul B comparativ cu grupul A (p<0,0001 ES).

În concluzie, semnele clinice de boală ca: absența pulsului femural şi hipertensiunea arterială sunt specifice pentru prezența coarctației de aortă. Asocierea leziunilor cardiace ca bicuspidia valvei aortice este mai frecventă în grupul B (38,46%) comparativ cu grupul A (9,09%).

Cuvinte cheie: coarctație aortică, tensiunea arterială, frecvența cardiacă, puls femural.

METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS STRAINS ISOLATED FROM AN ORTHOPAEDICS WARD AND AMBULATORY

M. BERCEANU VADUVA, H. PETRESCU, D. VERMESAN, F. TATU, A. LUPASCU, V. DUMITRASCU, ELENA HOGEA, DELIA MUNTEAN, MATILDA RADULESCU, MARCELA ADAMUT, CARMEN TATU, DANA VELIMIROVICI, DELIA BERCEANU VADUVA

University of Medicine and Pharmacy"Victor Babeş"Timişoara

ABSTRACT

Purpose: Staphylococcus aureus is one of the species most frequently isolated in in ambulatory, as well as in hospital environments, and can cause infections in both patients with immune deficiencies and healthy patients. It is one of the most pyogenic bacteria, capable of producing infections in any part of the human body. These are produced through the multiplication of the microorganisms (with direct invasion and tiss damage), or through release of toxins. The aim of the present paper is a comparative study of the sensitivity to antiinfectious chemotherapy drugs of S. aureus strains isolated from various pathological products collected from an orthopaedics ward and from ambulatory, and also to establish the resistance phenotypes of these strains. Of special interest was the study of the frequency of the Peni-R Meti-R (MRSA - Staphylococcus aureus methicillin-resistant) phenotype in the orthopaedics ward and ambulatory.

Material and methods: 951 S. aureus strains were studied: 60 isolated from 255 samples from the orthopaedics ward, and 891 isolated from 1620 samples from ambulatory. Testing of the antibiotic resistance was performed through Kirby-Bauer disk-diffusion method. Interpretative analysis was used to establish the resistance phenotypes of the germs.

Results: A high percentage of multiresistant strains was isolated, 21 strains (35.00%) isolated from the orthopaedics ward and 89 strains (9.99%) from ambulatory being identified as Peni-R Meti-R (MRSA) resistance phenotype.

Conclusions: The high frequency of Staphylococcus aureus Peni-R Meti-R (MRSA) resistance phenotype in the hospital environment explains the involvement of this germ in the ethiology of nosocomial infections. In order to limit the spread of these strains, especially within the hospital, enforcement of efficient nosocomial infections control techniques is necessary, as well as introducing policies for the rational antibiotic use.

Keywords: Staphylococcus aureus, MRSA, resistance phenotype.

INTRODUCTION

Staphylococcus aureus represents one of the most frequently isolated species both in ambulatory and in hospital; it can cause infections to patients with low infection resistance or to healthy, immune persons.

It is one of the most pyogenic bacteria, able to produce infections anywhere in the organism, starting with simple skin staphylococcic infections to infections with severe development. They are produced through the proliferation of microorganisms, with direct invasion and tissue destruction or through toxin release.

S. aureus provisionally colonizes mucous membranes and wet tegumentary areas. Almost 15-30% of normal adults are naso-pharynx bearers of S. aureus. For the medical staff and patients who were hospitalized for long time, the bearing rate may reach 40-80%, which explains the various nosocomial infections with this species.

The aim of the present paper is to test the drug sensitivity of S aureus strains isolated from various pathological productes collected from patients admitted in an orthopaedics ward and ambulatory, as well as to establish the resistance phenotypes of these strains. This way the best chemotherapeutic agent can be chosen and multi-drug resistance can be avoided. Of special interest was the study of the frequency of the Peni-R Meti-R (MRSA - Staphylococcus aureus methicillin-resistant) phenotype in the ambulatory and orthopaedics ward.

MATERIAL AND METHODS

951 S. aureus strains were studied: 60 isolated from 255 samples from the orthopaedics ward, and 891 isolated from 1620 samples from ambulatory. Testing of the antibiotic resistance was performed through Kirby-Bauer disk-diffusion method. The results were interpreted according to CLSI standard.

In order to test the sensitivity to antibiotics, the following drugs were used: penicillin (P), oxacillin (OX), kanamycin (K), tobramycin (TM), gentamicin (GM), erythromycin (E), clindamycin (CLI), vancomycin (VA), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT). Although some of the antibiotics included in panel for staphylococci susceptibility testing are not usually used in clinical practice, the testing panel was designed to detect the main mechanisms of resistance to antibiotics, and to allow identifying of the resistance phenotypes.

Interpretative analysis was used to establish the resistance phenotypes of the germs.

RESULTS AND DISCUSSIONS

Of the total 951 S. aureus strains studied, 891 were isolated from 1620 samples from ambulatory, and 60 were isolated from 255 samples from the orthopaedics ward.

Received September 2009. Accepted October 2009. Address for correspondence: Dr. Delia Berceanu, University of Medicine and Pharmacy"Victor Babeş"Timişoara, 2A Eftimie Murgu Square, 300041, Timisoara, Romania, e-mail: deliaberceanu@yahoo.com

Table I. Sample distribution

Origin	Nr.	%
Ambulatory	1620	86.40%
Orthopaedics	255	13.60%
TOTAL	**1875**	**100%**

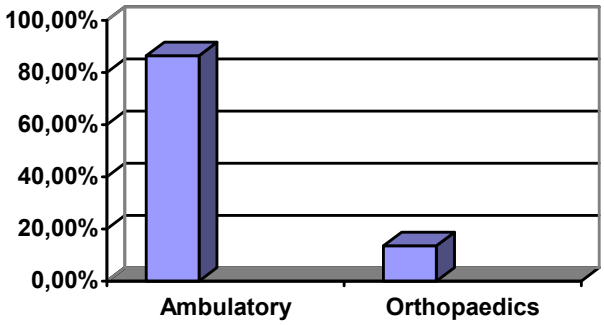


Fig. 1. Sample distribution

The specimens from which the S. aureus strains were isolated are presented in Tables II and III.

Table II. Specimens from which the S. aureus strains were isolated - ambulatory

Nr.	Specimens	Nr.	%
1.	Pharingeal swab	635	71.26
2.	Nasal swab	188	21.09
3.	Sputum	14	1.57
4.	Bronchial aspirates	3	0.33
5.	Ear secretion	15	1.68
6.	Pus	16	1.80
7.	Wound secretion	7	0.78
8.	Conjunctival swab	9	1.01
9.	Endocervical swab	4	0.45
	Total	**884**	**100%**

Table III. Specimens from which the S. aureus strains were isolated – orthopaedics

Nr.	Specimens	Nr.	%
1.	Wound secretion	46	76.66
2.	Pharingeal swab	6	10.00
3.	Sinovial fluid	3	5.00
4.	Blood	3	5.00
5.	Sputum	1	1.67
6.	Bronchial aspirates	1	1/67
	Total	**60**	**100%**

Interpretative analysis was used to establish the resistance phenotypes of the germs. Through the determination of the resistance phenotypes to antibiotics, information on the resistance mechanisms could be obtained and based on these mechanisms the antibiotics to which the tested bacteria was resistant could be detected.

The following phenotype resistance to beta-lactams were estabished: Peni-S Meti-S, Peni-R Meti-S, Peni-R Meti-R (MRSA).

Most of the isolated S. aureus strains proved to be Peni-R Meti-S (83.50% - ambulatory, 60% - orthopaedics). Only 6.51% of the ambulatory strais and 5% of the orthopaedics strains were sensitive to beta-lactams (Peni-S Meti-S). The MRSA presence in 9.99% of the ambulatory cases and 35.00% in the orthopaedics ward represents an alarm signal, because the MRSA strains are hospital strains, which are multi-resistant to antibiotics (Table IV and Figure 2). They present crossed resistance to all beta-lactams (penicillins, cephalosporins, carbapenemes, betalactamase inhibitors). The resistance extends to erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, quinolones, aminoglycosides, although the diffusimetric test may indicate a false sensitivity to these antibiotics.

Table IV. Resistance phenotypes to beta-lactams

Nr.	Phenotype	Ambulatory		Orthopaedics	
		Nr.	%	Nr.	%
1.	Peni-S Meti-S (wild type)	58	6.51	3	5.00
2.	Peni-R Meti-S	744	83.50	36	60.00
3.	Peni-R Meti-R (MRSA)	89	9.99	21	35.00
	Total	**891**	**100**	**60**	**100**

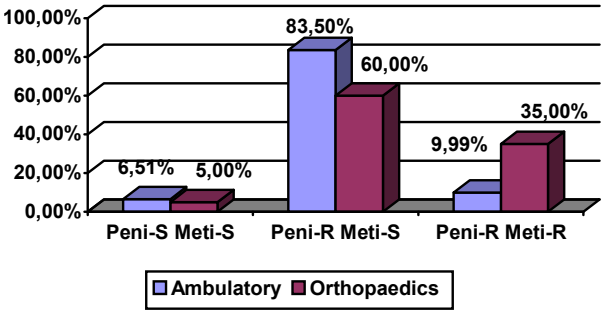


Fig. 2. Resistance phenotypes to beta-lactams

In order to determine the three resistance phenotypes to aminoglycosides (K, KT, KTG) kanamycin, tobramycin and gentamicin were used. Thus, susceptibility to amikacin and netilmicin can be readily deduced from the resistance or susceptibility profile to the three aminoglycosides tested (Table V and Figure 3).

The K phenotype (resistance to kanamycin) – encountered in 15.71% of the ambulatory strains and 11.66% of the orthopaedics strains, as well as the KT phenotype (resistance to kanamycin and tobramycin) – encountered in 0.45% of the ambulatory strains and 3.33% of the orthopaedics ward, are also predictive for resistance to amikacin; netilmicin being active.

Resistance to kanamycin, tobramycin and gentamicin (KTG phenotype) – encountered in 11.87% of the ambulatory strains and 15.00% of the orthopaedics ward, is predictive to resistance to amikacin and netilmicin.

Table V. Resistance phenotypes to aminoglycosides

Nr.	Phenotype	Ambulatory		Orthopaedics	
		Nr.	%	Nr.	%
1.	Wild type	642	72.05	42	70.00
2.	K	140	15.71	7	11.66
3.	KT	4	0.45	2	3.33
4.	KTG	105	11.78	9	15.00
	Total	**891**	**100**	**60**	**100**

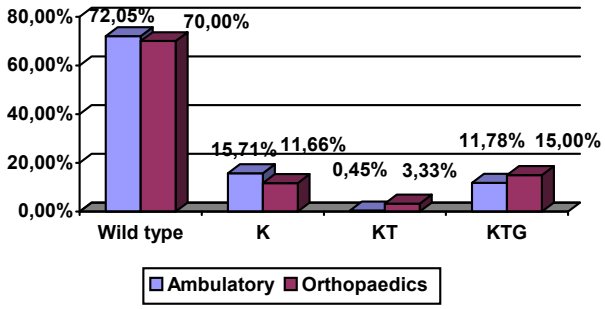


Fig. 3. Resistance phenotypes to aminoglycosides

In order to determine the resistance to MLS (macrolides, lincosamides, streprogramines) the association of erythromycin and clindamycin was tested using the diffusimetric method (Table VI and Figure 4).

The main resistance mechanism was the modifying of the ribosomal target, connected to the acquisition of the erm gene (which is responsible for resistance through ribosomal modification). The phenotypic expression of resistance can be inducible or constitutive: inducible MLS_B phenotype, constitutive MLS_B phenotype.

In the ambulatory 28.62% of the strains were resistant to the MLS, and 61.65% in the orthopaedics ward.

Table VI. Resistance phenotypes to MLS

Nr.	Phenotype	Ambulatory		Orthopaedics	
		Nr.	%	Nr.	%
1.	Wild type	636	71.38	23	38.33
2.	MLS_B inducilble	147	16.50	34	56.66
3.	MLS_B constitutive	89	9.99	2	3.33
4.	M (active efflux)	19	2.13	1	1.66
	Total	**891**	**100**	**60**	**100**

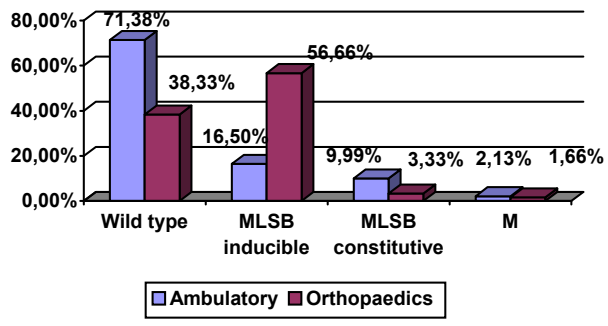


Fig. 4. Resistance phenotypes to MLS

In order to detect the resistance to fluoroquinolones, a single quinolone was used – ciprofloxacin; staphylococci being cross-resistant to all fluoroquinolones. Most strains were sensitive – 86.98% of the ambulatory strains and 61.67% of the othopaedics strains, and only few of them acquired resistance (13.02% - ambulatory, 38.33% - othopaedics) (Table VII and Figure 5).

Table VII. Resistance phenotypes to fluoroquinolones

Nr.	Phenotype	Ambulatory		Orthopaedics	
		Nr.	%	Nr.	%
1.	Wild type	775	86.98	37	61.67
2.	Resistant	116	13.02	23	38.33
	Total	**891**	**100**	**60**	**100**

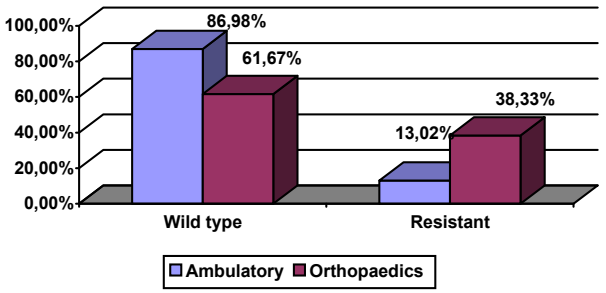


Fig. 5. Resistance phenotypes to fluoroquinolones

71.83% of the ambulatory strains and 56.66% of the orthopaedics strains were sensitive to trimethoprim-sulfamethoxazole, the rest of 28.16%, 43.33% respectively being resistant.

Table VIII. Resistance phenotypes to trimethoprim-sulfamethoxazole

Nr.	Phenotype	Ambulatory		Orthopaedics	
		Nr.	%	Nr.	%
1.	Wild type	640	71.83	34	56.66
2.	Resistant	251	28.17	26	43.33
	Total	**891**	**100**	**60**	**100**

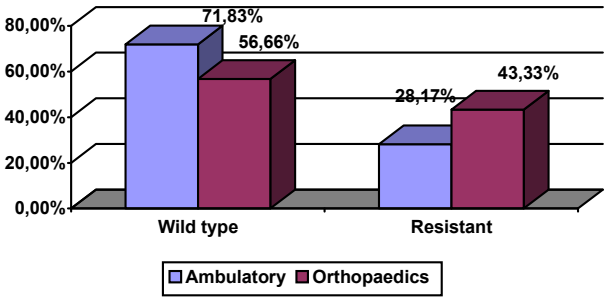


Fig. 6. Resistance phenotypes to trimethoprim-sulfamethoxazole

CONCLUSIONS

A high frequency, especially in the orthopaedics ward, was observed for the multiresistant S. aureus strains, which draws attention to the need for monitoring antibitic prescription. We believe that dvelopment of antibiotic resistance in these germs is caused by empirycal treatments, not based upon the antibiogram.

Presence of MRSA – 35.00 % in the orthopadics ward and 9.99% in the

ambulatory – represents an alarm signal, because the MRSA strains are hospital strains, multiresistant to antibiotics, with cross-resistance to all beta-lactams. Resistance is frequently extended to other antibiotic families.

The high frequency of Staphylococcus aureus Peni-R Meti-R (MRSA) resistance phenotype in the hospital environment explains the involvement of this germ in the ethiology of nosocomial infections. In order to limit the spread of these strains, especially within the hospital, enforcement of efficient nosocomial infections control techniques is necessary, as well as introducing policies for the rational use of antibiotics.

No strains resistant to vancomycin were described, but vancomycin must be used carefully to preserve its clinical utility. It is a back-up antibiotic, used as treatment of choise in infections determined by methicillin-resistant strains (MRSA).

REFERENCES

1. Angelescu M. Antibiotics therapy. *Ed. Medicala*, Bucharest, 1998.
2. Buiuc D, Negut M. Clinical microbiology IIIrd edition. *Ed. Medicala*, Bucharest, 2009.
3. Buiuc D. Medical microbiology: guide for medical study and practice. *Ed. "Gr. T. Popa"* Iasi, 2003.
4. Campbell CW et al. Campbell's Operative Orthopaedics, Mosby, Missouri, 1998.
5. Epps HC Jr et al. Complications In Orthopaedic Surgery. J.B. Lippincott Company, Philadelphia, 1986.
6. Greenwood D. Antimicrobial Chemotherapy, Oxford, University Press, 1989.
7. Jehl F, Chomarat M, Weber M, Gerard A. From antibiogram to prescription. *Ed. Stiintelor Medicale*, Bucharest, 2004.
8. Lennette EH, Balows A, Hausler WJ, Truant JP. Manual of Clinical Microbiology, 4th ed, *American Society for Microbiology*, Washington, DC, 1985.
9. Licker M, Moldovan R et al. Special microbiology volume I - Bacteriology. *Ed. Eurostampa*, Timisoara, 2008.
10. Lorian V. Antibiotics in clinical medicine, 2nd ed, Williams and Wilkins, Baltimore, 1986.
11. Mandell GL, Douglas RG, Bennett JE. Principles and practice of infectious disease. Animicrobial therapy, Churchill Livingstone, New York, 1992.
12. Moldovan R et al. Microbiology – Practical laboratory support, lito UMFT, 2002.
13. Murray RP, Kobayashi SG, Pfaller AMI, Rosenthal SK. Medical Microbiology, Wolfe Imprint., 1994.
14. Nechifor M, Vlase C. Progresses and perspectives in antibacterial chemotherapy. *Ed. Viata Medicala Romaneasca*, 2001.
15. Prundeanu A, Vermesan H, Prundeanu H. Polytraumatisms. *Ed. Mirton*, Timisoara, 2001.
16. Vermesan H et al. Orthopedics. *Ed. Mirton*, Timisoara, 2001.

REZISTENȚA LA METICILINĂ A TULPINILOR DE STAPHYLOCOCCUS AUREUS IZOLATE DINTR-O SECȚIE DE ORTOPEDIE ŞI DIN AMBULATOR

REZUMAT

Introducere: Staphylococcus aureus reprezintă una din speciile izolate cel mai frecvent atât în ambulator, cât şi în mediul spitalicesc, ce poate determina infecții atât la persoanele cu rezistență antiinfecțioasă scăzută, cât şi la persoanele sănătoase. Este una dintre cele mai piogene bacterii, capabilă de a produce infecții cu orice sediu în organism. Ele se produc prin proliferarea microorganismelor (cu invazie directă şi distrugerea țesuturilor) sau prin eliberarea de toxine.

Scopul prezentei lucrări este reprezentat de studiul comparativ sensibilității la chimioterapicele antiinfecțioase a tulpinilor de Staphylococcus aureus izolate din diverse produse patologice provenite dintr-o clinică de ortopedie şi din ambulator, precum şi stabilirea fenotipurilor de rezistență în care se încadrează aceste tulpini. S-a urmărit incidența tulpinilor de Staphylococcus aureus Peni-R Meti-R (MRSA - Staphylococcus aureus meticilino-rezistent) în secția de ortopedie şi în ambulator.

Material şi metodă: S-au luat în studiu 951 tulpini de Staphylococcus aureus: 60 izolate din 255 probe provenite din clinica de ortopedie şi 891 izolate dintr-un număr de 1620 probe din ambulator. Testarea sensibilității la antibiotice s-a realizat prin metoda difuzimetrică Kirby-Bauer. Prin analiza interpretativă a antibiogramelor s-a făcut încadrarea germenilor în fenotipuri de rezistență.

Rezultate: S-a izolat un procent crescut de tulpini de Staphylococcus aureus cu multirezistență la antibiotice, 21 tulpini (35,00%) izolate din clinica de ortopedie şi 89 tulpini (9,99%) provenite din ambulator încadrându-se în fenotipul de rezistență Peni-R Meti-R.

Concluzii: Frecvența crescută a tulpinilor Staphylococcus aureus Peni-R Meti-R (MRSA) din spital explică implicarea acestui germen în etiologia infecțiilor nosocomiale. Pentru limitarea răspândirii în special în mediul spitalicesc a acestor tulpini, se impune instituirea unor măsuri eficace de control al infecțiilor nosocomiale şi introducerea unor politici de utilizare rațională a antibioticelor.

Cuvinte cheie: Staphylococcus aureus, MRSA, fenotip de rezistență.