ROMANIAN ARCHIVES

OF

MICROBIOLOGY

AND

IMMUNOLOGY

Founded by
PROFESSOR ION CANTACUZINO
in 1928

VOLUME 74 - Issue 3-4
July - December 2015

Published quarterly
by
CANTACUZINO INSTITUTE BUCHAREST

TOTAL PUBLISHING HOUSE

ROMANIAN ARCHIVES
OF
MICROBIOLOGY
AND
IMMUNOLOGY

Print ISSN 1222-3891

INDEXED IN MEDLINE

TOTAL PUBLISHING HOUSE

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Cantacuzino National Institute of Research

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Romanian Archives of Microbiology and Immunology

MECHANISMS OF RESISTANCE TO CIPROFLOXACIN AND GENETIC DIVERSITY OF *ESCHERICHIA COLI* STRAINS ORIGINATING FROM URINE CULTURES PERFORMED FOR ROMANIAN ADULTS

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ABSTRACT

Urinary tract infections (UTI) with *Escherichia coli* are among the most common infections presenting in general practice. Fluoroquinolones (FQs) are relied on for their empirical therapy but recent reports indicate a concerning increase in the percentage of FQ-resistant *E. coli* isolates in many countries, including Romania.

Sixty *E. coli* strains with ciprofloxacin resistance and cephalosporin susceptibility isolated from urine specimens of non-hospitalized patients during a five-month period (October 2014 – February 2015) were further analyzed to determine the molecular basis of FQ resistance (i.e. mutations in chromosomal *gyrA*, *gyrB*, *parC* genes and presence of plasmid-borne *qnrA*, *qnrB*, qnrS, and *aac(6')-Ib-cr* genes), the phylogenetic background (i.e. phylogenetic groups A, B1, B2, C, D, E, F or clade I), O25b/ST131 status, and genetic relatedness inferred from the *XbaI* pulsed-field gel electrophoresis (PFGE) profiles as a measure of isolate-specific genetic composition.

The PCR-based phylotyping showed that most strains were assigned to non-B2 phylogenetic groups (i.e. group A/21 strains, group B1/14 strains, group B2/10 strains, group C/8 strains, group D/3 strains, group F/4 strains). Already described chromosomal mutations associated to FQ resistance were found, the strains being double *gyrA* mutants (i.e. Ser83Leu, Asp87Asn) with one or two *parC* mutations (e.g. Ala56Thr, Ser80Ile, Glu84Gly). Seven percent of the strains harboured plasmid-borne genes *qnrS1* (2 strains) and *aac(6')-Ib*-cr (2 strains). Based on the PCR results, 15% of the strains were members of the O25b/ST131 clone and possessed the *gyrA*/*parC* allele combination which is considered as hallmark of *H30* subclone. PFGE genotyping revealed a genetically diverse population of FQ-resistant *E. coli*. ST131 strains displayed more homogeneous PFGE profiles than non-ST131. The ST131 cluster extended to 77.74% similarity versus 60% overall.

These findings underscore the need for ongoing surveillance to capture the complexity of the emerging population of FQ-resistant strains disseminated across our community.

REZUMAT

Infecţiile urinare cu *Escherichia coli* sunt printre cele mai frecvente infecţii cu care se confruntă practicienii. Terapia lor empirica se bazeaza pe fluorochinolone (FC), dar rapoarte recente din multe tari, inclusiv România, indică o creştere importantă a procentului de izolate *E. coli* rezistente.

Un număr de 60 de tulpini de *E. coli* rezistente la ciprofloxacin şi sensibile la cefalosporine, izolate din probe de urină de la pacienţi trataţi în ambulator într-o perioadă de 5 luni (octombrie 2014 – februarie 2015), au fost ulterior analizate pentru a determina baza moleculară a rezistenţei la FC (mutaţii cromozomale în genele *gyrA*, *gyrB*, *parC* şi prezenţa genelor plasmidice *qnrA*, *qnrB*, qnrS şi *aac(6')-Ib-cr*), fondul filogenetic (grupurile filogenetice A, B1, B2, C, D, E, F sau clada I), statusul O25b/ST131 şi înrudirea genetică rezultată din profilurile PFGE de macrorestricţie cu enzima *XbaI*.

Rezultatele de filotipizare prin PCR au arătat că majoritatea tulpinilor s-au distribuit în grupurile filogenetice non-B2 (grup A/21 tulpini, grup B1/14 tulpini, grup B2/10 tulpini, grup C/8 tulpini, grup D/3 tulpini, grup F/4 tulpini). Au fost identificate mutaţii cromozomale cunoscute, asociate rezistenţei la FC, toate tulpinile având o dubla mutaţie în gena *gyrA* (Ser83Leu, Asp87Asn) şi una sau două mutaţii în gena *parC* (Ala56Thr, Ser80Ile, Glu84Gly). Un procent de 7% dintre tulpini au prezentat şi genele plasmidice *qnrS1* (2 tulpini), respectiv *aac(6')-Ib-cr* (2 tulpini). Pe baza rezultatelor PCR, 15% dintre tulpini s-au dovedit parte a clonei O25b/ST131 având combinatia alelica *gyrA*/*parC* specifică subclonei *H30*. Genotipizarea prin PFGE a evidenţiat o populaţie de tulpini de *E. coli* rezistente la FC diversă din punct de vedere genetic. Tulpinile ST131 au prezentat profile PFGE mai omogene decât cele non-ST131, grupul lor extinzându-se până la 77.7% similaritate, faţă de similaritatea globală de 60%.

Aceste date subliniază necesitatea unei supravegheri continue pentru a surprinde complexitatea populaţiei emergente de tulpini rezistente la FC diseminată în comunitatea noastră.

Keywords: Ciprofloxacin resistance, *E. coli* phylogenetic groups, *E. coli* molecular characterization

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INTRODUCTION

Urinary tract infection (UTI) with *Escherichia coli* is one of the most common infections presenting in general practice [1]. Most UTI episodes are treated with antibiotics because many clinicians and patients believe that the clinical improvement is faster achieved in this way. In outpatients with community-associated infections, empirical treatment is usually preferred to culture directed therapy because the latter involves more time and money, but in the last years the empiric decisions are challenged by increasing antibiotic resistance levels observed for many uropathogens including *E. coli* [2]. Of particular concern is *E. coli* resistance to fluoroquinolones (FQs), since the use of these agents as first-line treatment of uncomplicated cystitis is encouraged by many physicians. The most common mechanism of FQ resistance in *E. coli* is represented by alterations occurring in genes encoding for DNA gyrase (i.e. *gyrA* and *gyrB*) and topoisomerase IV (i.e. *parC* and *parE*), the FQ primary and secondary target enzymes in Gram-negative bacteria, respectively [3]. Mutations that affect efflux pumps or cell permeability, and plasmid-borne factors may also contribute to resistance [4].

Molecular studies focusing on the *E. coli* population associated with extraintestinal infections are indicating a rapid and global spread of a specific FQ resistance-associated clonal group, defined by multilocus sequence typing (MLST) as ST131 clone [5]. Specifically, FQ resistance is associated predominantly with a ST131 subclone, designated *H*30 because its members possess the allele 30 of the gene encoding type 1 fimbrial adhesin (*fimH*). The H30 strains that express concurrent resistance to FQs and extended-spectrum cephalosporins due to extended-spectrum-beta-lactamase (ESBL) CTX-M-15 form another subclone, named H30-Rx [6].

According to the latest official reports issued by the European Centre for Disease Prevention and Control (ECDC) for year 2013, Romania was among the countries with more invasive infections due to *E. coli* strains with resistance to FQ (31%) than to the third-generation cephalosporins (22.8%) or aminoglycosides (14.8%) [http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2013]. A high FQ consumption was also observed among the Romanian population but the available report did not discriminate between the community and hospital care sectors [http://www.who.int/drugresistance/documents/surveillancereport/en/(2014)]. The information was concerning, the absence of solid knowledge of the characteristics of autochthonous FQ-resistant *E. coli* population making it difficult to determine if this phenomenon was mainly due to the conversion of susceptible strains into resistant ones under an increasing antibiotic pressure or rather to the dissemination of already resistant clones.

This study focused on a set of *E. coli* urinary isolates from community source specimens aiming to determine the genetic basis of their fluoroquinolone resistance, phylogenetic background, O25b/ST131 status, and genetic relatedness as inferred from pulsed-field gel electrophoresis (PFGE) typing results.

MATERIALS AND METHODS

E. coli isolates

During a five-month period (October 2014 – February 2015), a private clinical laboratory in Bucharest providing clinical microbiological services for the community clinics registered 320 *E. coli*-positive urine specimens. Sixty strains originating from these specimens, selected according to the combined fluoroquinolone and third generation cephalosporin phenotype, i.e. ciprofloxacin and/or norfloxacin resistance and third-generation cephalosporin susceptibility, were further studied at molecular level at Cantacuzino National Institute of Research (formerly Cantacuzino National Institute of Research-Development for Microbiology and Immunology).

PCR-based phylotyping

The assignment of the *E. coli* isolates to the phylo-groups A, B1, B2, C, D, E, F, and clade I was done using a revisited protocol of Clermont *et al.* [7].

PCR for identification of isolates belonging to O25b-ST131 clone

The detection of isolates belonging to O25b-ST131 clone was performed by an allele-specific PCR assay targeting the *pabB* gene which was previously described by Clermont *et al*. [8].

Screening for mutations in the quinolone-resistance determining regions (QRDRs) of genes *gyrA*, *gyrB*, and *parC*

The amplification of DNA fragments including the QRDRs of *gyrA*, *gyrB*, and *parC* genes was performed using primers with published sequences which generated PCR products of 648 bp (*gyrA*) [9],

447 bp (*gyrB*) [10], and 418 bp (*parC*) [11], respectively. After purification with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), the PCR products were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the same primers used in the PCR assays. The sequencing products were further purified with DyeEx 2.0 Spin Kit (Qiagen) and analyzed using an ABI 3130 platform (Applied Biosystems). DNA sequences obtained were compared with the antibiotic susceptible strain *E. coli* BL21 (DE3) (GenBank accession no. NC_012971.2).

Screening for plasmid-encoded FQ resistance genes *qnrA*, *qnrB*, *qnrS,* and *aac(6')-Ib*-cr

Plasmid-encoded FQ resistance genes *qnrA*, *qnrB*, and *qnrS* were detected using a previously published multiplex PCR-based protocol [12] and primers which generated amplicons of 580 bp, 264 bp, and 428 bp, respectively. The amplicons were further sequenced using the PCR primers in order to detect the *qnr* gene variants.

The presence of *aac(6')-Ib-cr* gene was detected by using previously published primers that amplified all known *aac(6')-Ib* variants, generating a 428-bp product [13], which was further sequenced to detect the *cr* variant.

Pulsed-field gel electrophoresis analysis

All isolates underwent pulsed-field gel electrophoresis (PFGE) analysis of *Xba*I-restricted total DNA according to the PulseNet protocol [http://www.pulsenetinternational.org/protocols/]. By using BioNumerics (Applied Maths) and band-based Dice similarity coefficients, pulsotype designations were assigned at the ≥90% profile similarity level, corresponding to an approximately 3-band difference [14].

RESULTS AND DISCUSSION

To better understand the basis of FQ resistance and document a potential clonal component to this resistance, we performed a molecular epidemiological analysis of ciprofloxacin-resistant *E. coli* strains recovered from UTIs, infections with a high prevalence among predominantly healthy individuals from community. We excluded from investigation the strains with concurrent resistance to cephalosporins in order to exclude the contribution of strains with this type of resistance to the clonal component of FQs emergence.

We rapidly assessed the phylogenetic background of the set of ciprofloxacin-resistant *E. coli* by using a protocol which allowed the delineation of seven phylogenetic groups belonging to *E. coli sensu stricto* (i.e. A, B1, B2, C, D, E, and F) and one corresponding to *Escherichia* clade I. All the studied strains were typeable, the PCR results indicating strains derived from groups A (21 isolates), B1 (14 isolates), B2 (10 isolates), C (8 isolates), D (3 isolates), and F (4 isolates). To our knowledge, this is the first report on autochthonous isolates belonging to newly described phylogenetic groups C and F, considered as closely related to B1 and B2, respectively. An Iranian study found that 39% of 140 *E. coli* isolates from UTIs belonged to groups C, E, F, and clade I strains [15], whereas an Algerian study reported that 13% of a set of 150 community-acquired uropathogenic *E. coli* belonged to phylogenetic groups C, F and clade I [16]. Of note, the Iranian report also indicated that 27% of the urinary strains could not be assigned to a known phylogenetic group suggesting as possible explanation the presence of extremely rare groups or large-scale recombination events.

Phylogenetic group B2, and to a lesser extent group D, are traditionally recognized as sources of extraintestinal pathogenic *E. coli* (ExPEC) [17]. Using the phylogenetic affiliation as the sole criterion to predict virulence capability, 78% of the urinary strains investigated would not have qualified as uropathogens which is consistent to previous reports showing that ciprofloxacin resistance usually occurred in low-virulence phylogroups [18, 19].

The ST131 *E. coli* clone had a dramatic expansion worldwide after it acquired FQ resistance. Interested in the evaluation of the contribution of this clone to the FQ resistance among the local strains we used a specific PCR-based assay as a rapid alternative to MLST and identified 9 ST131-positive strains. This finding cumulated with previous reports [20, 21], documenting the emergence of ST131 clone among invasive CTX-M-15-producing *E. coli* co-resistant to FQ, highlighted the circulation of multiple ST131 lineages.

As expected, FQ resistance of autochthonous *E. coli* strains was mainly the result of mutations in the antimicrobial targets, all the urinary isolates being identified as double *gyrA* mutants with one or two additional mutations in the *parC* but none in *gyrB* gene. It is worth noting that all ST131 isolates displayed a similar *gyrA*/*parC* allele combination that is considered a hallmark of the *H30* subclone [22]. In some isolates, this dominating chromosomal

mechanism was combined with the plasmid-mediated one. Thus, three of the seven resistance genotypes detected among ciprofloxacin-resistant isolates, combined *gyrA* and *parC* replacement mutations with *qnrS1* or *aac(6')-Ib-cr* genes (Table 1). The *qnrS1* gene encodes QnrS1, one of the proteins of the larger Qnr family, known to protect DNA gyrase and type IV topoisomerase from quinolone inhibition [4], whereas *aac(6')-Ib-cr* encodes a variant of aminoglycoside acetyltransferase that inctivates ciprofloxacin and norfloxacin [23]. Plasmid-mediated quinolone resistance in *E. coli* strains was first reported in Europe only in 2003, when one *E. coli* isolate out of the 297 *E. coli* isolates tested at the Bicêtre Hospital in Paris was *qnr*-positive [24]. The detection of the first Romanian *qnrS*-positive *E. coli* isolate was reported in 2009 [25] and our results confirmed that the plasmid-mediated mechanisms played an important role in the spread of FQ resistance among the autochthonous *E. coli* isolates.

The *aac(6')-Ib-cr* gene was described in both ESBL-producing *E. coli* strains [26], including those belonging to ST131 clone [27] and non-ESBL-producing ones [28, 29]. Studies investigating *E. coli* plasmids encoding CTX-M-15 β-lactamases described *aac(6')-Ib-cr* gene linked to $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ on plasmids belonging to incompatibility group FII [28, 29]. Various molecular epidemiology studies indicated that the prevalence of *E. coli* strains harboring *aac(6')-Ib-cr* gene increased continuously in all the regions where emerged [4]. Therefore, this aspect should be taken into account while screening for further autochthonous FQ resistant strains.

In order to determine their genetic diversity, we subjected all ciprofloxacin-resistant *E. coli* strains to standardized PFGE analysis. All but five strains were typeable. The dendrogram constructed within BioNumerics software showed that the 90% criterion resolved 46 pulsotypes, each accounting for one to three isolates. There were 38 single-isolate pulsotypes, seven two-isolate pulsotypes, and one three-isolate pulsotype. The ST131 strains were distributed in 5 pulsotypes and exhibited more homogeneous profiles than non-ST131 strains, the ST131 cluster extended to 77.74% similarity versus 60% overall.

On the basis of these findings we can conclude that the emergence of the FQ resistance of autochtonous *E. coli* strains is mostly due to spontaneous mutations as a result of FQ exposure and plasmids are presumed to also contribute to such phenotype but to a lesser extent. The FQ-resistant *E. coli* population circulating in epidemiologically unrelated patients from community is genetically diverse. However, the identification of ST131 members within it reinforces the message that the threat of the expansion of a clonal reservoir of FQ resistant strains must be carefully considered and an ongoing surveillance to capture the complexity of the emerging population of FQ-resistant strains disseminated across our community is needed.

Acknowledgements

This work received financial support through the project entitled "CERO – Career profile: Romanian Researcher", grant number POSDRU/159/1.5/S/135760, co-financed by the European Social Fund for Sectorial Operational Programme Human Resources Development 2007-2013".

Table 1. **Genotypes of fluoroquinolone resistance detected in the 60 ciprofloxacin-resistant *E. coli* urinary isolates in relationship with the phylogenetic background of the isolates**

FQ resistance genotype	No. of isolates	Phylogenetic group*
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ser80Ile}}$	34	A, B1, B2, C, E, F
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ser80Ile}}$; *qnrS1*	2	A, B1
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ser80Ile}}$; *aac(6')-Ib-cr*	1	C
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ser80Ile, Glu84Val}}$	10	A, B2*
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ser80Ile, Glu84Gly}}$	6	A, B1, C, F
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ser80Ile, Glu84Val;}}$ *aac(6')-Ib-cr*	1	B2*
$gyrA_{\text{Ser83Leu, Asp87Asn}}$; $parC_{\text{Ala56Thr, Ser80Ile}}$	7	A, C, E

Legend for amino acid code: Ala, alanine; Asn, asparagine; Asp, aspartate; Glu, glutamate; Gly, glycine; Ile, isoleucine; Leu, leucine; Ser, serine; Val, valine; Thr, threonine.

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INHIBITION OF VIRULENCE FACTORS OF *PSEUDOMONAS AERUGINOSA* BY DICLOFENAC SODIUM

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ABSTRACT

Resistance of *Pseudomonas aeruginosa* to antibiotics is a major problem. Targeting virulence factors is an alternative option to avoid the emergence of resistance to antibiotics. The effect of sub-inhibitory concentration of diclofenac sodium on the production of virulence factors of *P. aeruginosa* was investigated. The virulence factors included protease, haemolysin, pyocyanin and pyoverdin, in addition to pathogenic behaviors such as swimming and twitching motilities and biofilm formation. Diclofenac sodium showed significant inhibition of virulence factors as compared to the control. Diclofenac sodium decreased twitching and swimming motilities by 29.27% and 45.36%, respectively. The percentage of inhibition of pyocyanin by diclofenac sodium was 42.32%. On the other hand, pyoverdin was inhibited to a lesser extent (36.72%). Diclofenac sodium reduced protease by 52.58% and biofilm formation by 58.37%. Moreover, haemolytic activity in the presence of diclofenac sodium was 15.64% as compared to the control (100% haemolytic activity).

The inhibitory activities may be due to inhibition of quorum sensing that regulates the expression of virulence factors. This study suggests the potential for the use of diclofenac sodium as an anti-virulence agent in the treatment of *Pseudomonas aeruginosa* infections.

REZUMAT

Rezistenţa *Pseudomonas aeruginosa* la antibiotice reprezintă o problemă majoră. Ţintirea factorilor de virulenţă constituie o opţiune alternativă pentru evitarea apariţiei rezistenţei la antibiotice. S-a investigat efectul concentraţiei sub-inhibitorii de diclofenac sodic asupra producţiei de factori de virulenţă la *P. aeruginosa*. Factorii de virulenţă au inclus proteaza, hemolizina, piocianina şi pioverdina, pe lângă comportamentele patogenice, cum ar fi „swimming and twitching motilities" şi formarea de biofilm. Diclofenacul sodic a arătat o capacitate semnificativă de a inhiba factorii de virulenţă în comparaţie cu martorul. Diclofenacul sodic a diminuat „swimming and twitching motilities" cu 29,27% şi, respectiv, 45,36%. Procentul inhibării piocianinei de către diclofenacul sodic a fost de 42,32%. Pe de altă parte, pioverdina a fost mai puţin inhibată (36,72%). Diclofenacul sodic a redus proteaza cu 52,58% şi formarea de biofilm cu 58,37%. Mai mult chiar, activitatea hemolitică în prezenţa diclofenacului sodic a fost de 15,64% în comparaţie cu martorul (100% activitate hemolitică).

Activităţile inhibitorii se pot datora inhibării „quorum sensing" (detectarea cvorumului) care reglează expresia factorilor de virulenţă. Acest studiu sugerează posibilitatea utilizării diclofenacului sodic ca agent anti-virulenţă în tratamentul infecţiilor cu *P. aeruginosa.*

Keywords: *Pseudomonas aeruginosa*, virulence factors, inhibition, diclofenac sodium

INTRODUCTION

Pseudomonas aeruginosa is a frequent nosocomial pathogen that causes urinary tract infection. It is highly resistant to antibiotics and the resistance is enhanced by its ability to form biofilms [1]. The high resistance of *Pseudomonas aeruginosa* led to the search for new methods to fight infections. Targeting virulence factors is one approach that can enhance the immune system to eradicate the infectious agent and avoid the emergence of antibiotic resistance at the same time [2, 3]. Quorum sensing (QS) regulates the expression of virulence factors. Quorum sensing is a mechanism of cell-to-cell communication between bacterial cells by means of autoinducers or signaling molecules that reflect the bacterial cell density [4]. *N*-acylhomoserine lactones (AHLs) are the autoinducers in *P. aeruginosa* [5]. *P. aeruginosa* produces several virulence factors including pyocyanin, haemolysin, alkaline protease and the siderophore pyoverdine. Motility contributes to the virulence of *Pseudomonas aeruginosa* [6-9]. It also

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produces biofilm that plays an important role in pathogenesis and resistance. The resistance of biofilm cells may reach up to 1000 times that of planktonic cells [10, 11].

Non-steroidal anti-inflammatory drugs (NSAIDs) were found to have good antimicrobial activities [12]. Diclofenac sodium exhibited a potent antimicrobial activity against *P. aeruginosa* and also showed inhibitory activity against biofilm formation [13]. This study aimed to investigate the inhibitory activities of diclofenac sodium on the production of virulence factors by *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Media and Chemicals

Tryptone soya broth and Mueller Hinton broth were purchased from Oxoid (Hampshire, UK), Luria-Bertani (LB) broth, LB agar, peptone and tryptone from Lab M Limited (Lancashire, United Kingdom), azocasein from Sigma (St. Louis, USA) and diclofenac sodium from Novartis Pharma, Cairo, Egypt. Other chemicals were of pharmaceutical grade.

Bacterial Strains

One clinical *Pseudomonas aeruginosa* isolate was isolated from a patient admitted to Zagazig University Hospital with a urinary tract infection. It was identified by Gram-staining, growth on MacConkey agar, oxidase test, green pigmentation on nutrient agar, motility, growth at 42°C according to Koneman *et al*. [14].

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MICs) of diclofenac sodium was determined by the broth microdilution method according to the Clinical Laboratory and Standards Institute Guidelines [15].The tested strain was incubated overnight in tryptone soya broth (TSB) and the turbidity was adjusted to match the turbidity of 0.5 McFarland Standard. The bacterial suspension was diluted with Mueller-Hinton broth to have an approximate cell density of 10^6 CFU/ml. The bacterial suspension was added in aliquots of 50 µl to the wells of a microtiter plate to which aliquots of 50 µl of double the required dilutions of the tested agent in Mueller-Hinton broth were added. After incubation of the plates at 37ºC for 20 h, the MIC was calculated as the lowest concentration of the tested agent that inhibited the visible growth in the wells.

Quantitative Assessment of Biofilm By The Spectrophotometric Method

To test the ability of *P. aeruginosa* strain to form biofilm, the modified method of Stepanovic *et al*. [16] was used. An inoculum of 1×10^6 CFU/ml was prepared by dilution of an overnight culture of the tested strain with TSB. Aliquots of 100 µl of the prepared suspension were delivered to the wells of 96-well sterile microtiter plates with rounded bottom and the plates were incubated for 24 h at 37°C. To remove the planktonic cells, the contents of the microtiter plates were gently removed and the wells were washed 3 times with sterile phosphate buffered saline (PBS, pH 7.2).

The adherent bacterial cells were fixed with addition of aliquots of 100 µl of 99% methanol for 20 minutes. Methanol was removed, the wells were stained with aliquots of 100 µl crystal violet (1%) for 20 minutes and any excess dye was removed by washing with distilled water. The plates were air-dried and the bound dye was dissolved by aliquots of 80 µl of 33% glacial acetic acid. The optical densities were measured with a spectrofluorimeter (Biotek, USA) at 590 nm.

Measurements were performed in triplicate and repeated 3 times. The cut-off optical density (ODc) was calculated as three times standard deviations above the mean OD of the negative control. The tested strains were classified according to the criteria of Stepanovic *et al*. (2007) into non-biofilm producer (OD ≤ ODc), weak biofilm producer (OD >ODc, but ≤ 2x ODc), moderate biofilm producer (OD>2x ODc, but ≤ 4x ODc), and strong biofilm producer (OD> 4x ODc).

Swimming and Twitching Motilities Assay

Swimming and twitching assays were performed according to Rashid and Kornberg [17]. Swimming agar plates composed of 1% tryptone, 0.5% sodium chloride and 0.3% agar and containing sub-inhibitory concentration of diclofenac sodium were prepared. Control plates without the tested agent were also prepared. The plates were surface inoculated in the center with 5µl of diluted overnight cultures in tryptone broth and incubated for 24h at 37°C. The diameters of swimming zones were measured.

For twitching assay, LB agar plates (1%) were stab-inoculated with 2µl of the prepared culture and incubated at 37°C for 48h. The agar was removed and the plates were left to dry. The twitching zones were measured. The experiment was repeated three times and the results were averaged.

Assay of Virulence Factors

The tested strain was grown overnight in LB broth with and without sub-MIC of the tested agent at 37°C and the cell free supernatant was separated by centrifugation at 8500 *g* for 15 minutes and used for assay of virulence factors [18].

Protease Assay

Protease production was determined qualitatively on skim milk agar plates in the presence and absence of sub-inhibitory concentration of diclofenac sodium according to Huber *et al.* [19] with some modifications. LB agar plates containing 5% skim milk were inoculated on the surface with 5 µl of overnight suspension of *P.aeruginosa* in LB broth. After incubation for 24h at 37°C, the clear zone around the microorganism was measured. The azocasein assay was used to estimate the proteolytic activity according to Kessler *et al.* [20]. One ml of azocasein (0.3%) in 0.05M Tris-HCl and 0.5M $CaCl_2$ (pH 7.5) was added to 150 µl of the prepared free cell culture supernatant, and the mixture was incubated for 15 minutes at 37°C. Trichloroacetic acid (10%, 0.5 ml) was added to stop the reaction and the precipitated azocasein was removed by centrifugation. The absorbance of the supernatants was measured at 400 nm using Biotek Spectrofluorimeter (Biotek, USA).

Pyocyanin Assay

The assay was performed according to Essar *et al*. [21] and Ra'oof and Latif [22]. The tested strain was grown overnight in LB broth and diluted to reach OD_{600} of 0.02. King A media composed of peptone 2%, K_2SO_4 1% and $MgCl_2$ 0.14% was inoculated with the diluted culture in the absence and presence of diclofenac sodium. The cultures were incubated at 37°C for 48h and the absorbance of pyocyanin was measured at 520 nm using Biotek Spectrofluorimeter (Biotek, USA). Pyocyanin concentration was calculated from the formula (pyocyanin concentration in µg/ml = OD_{520} X 17.072). The experiment was performed in triplicate and the mean concentration was calculated.

Haemolysin Assay

To determine the inhibition of haemolysin by diclofenac sodium, the modified method of Rossignol *et al*. [23] was used. Cell free supernatant (0.5 ml) was mixed with 0.7 ml of fresh 2% v/v erythrocytes suspension in saline and incubated at 37°C for 2h. The haemoglobin release was measured at 540 nm after centrifugation at 2500 *g* for 5 minutes at 4°C. The haemoglobin release was compared with positive control (erythrocytes completely lysed with 0.1% SDS) and negative control (erythrocytes incubated in LB broth). Percentage haemolysis was calculated from the formula:

$$\% \text{ hemolysis} = [X\text{-}B/T\text{-}B] \times 100,$$

where X is the treated and untreated samples, B is the negative control and T is the positive control. The haemolysis by treated cultures was expressed as % compared to haemolysis by untreated culture. The experiment was made in triplicate.

Pyoverdin Assay

The cell free supernatants were diluted 10-fold in Tris-HCl buffer (pH 7.4). Aliquots of 100 µl were added to 96-well microtiter plates in ice and the measurement of pyoverdin concentration was based on the fluorescence of the test supernatant at an excitation wavelength of 405 nm and an emission wavelength of 465 nm using Biotek Spectrofluorimeter [24].

Statistical Analysis

The effects of diclofenac sodium on virulence factors of *P. aeruginosa* were compared by One Way ANOVA followed by Bonferroni's Multiple Comparison Test, Graph Pad Prism 5. *P* values <0.05 were considered statistically significant.

RESULTS

Identification of *Pseudomonas Aeruginosa*

The tested isolate was identified as *Pseudomonas aeruginosa.* It was Gram-negative bacilli, oxidase positive, motile, produced green pigmentation on nutrient agar and could grow at 42°C.

Determination of Antibacterial Activity

Diclofenac sodium could inhibit the growth of the tested strain of *P. aeruginosa* at 2 mg/ml. The inhibitory effects against *P. aeruginosa* virulence factors were tested at a concentration equivalent to 1/4 MIC.

Assessment of Biofilm Formation

According to the criteria of Stepanovic *et al*. [16], the tested strain was strong biofilm forming because its OD was higher than 4 times ODc. ODc was calculated as 0.068.

Inhibition of Twitching and Swimming Motilities

Diclofenac sodium caused a significant decrease in twitching and swimming motilities as compared to the control ($P< 0.05$). Diclofenac sodium decreased twitching and swimming motilities by 29.27% and 45.36%, respectively (Figs. 1-3).

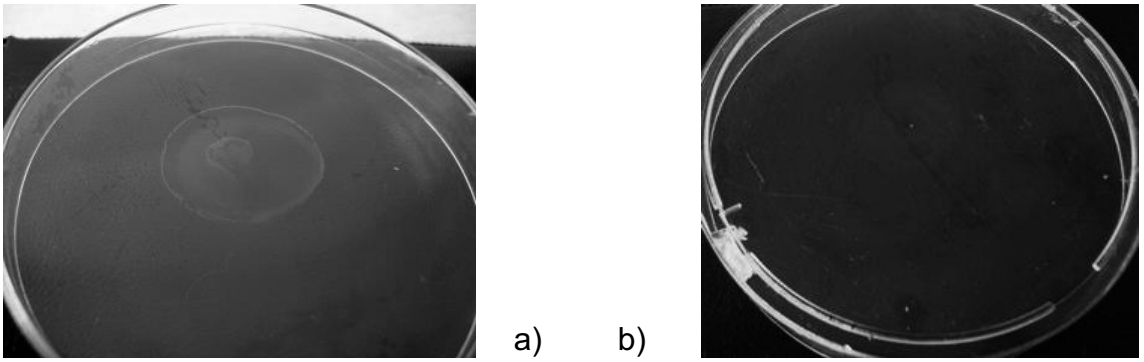

Fig. 1. Inhibition of twitching motility by sub-MICs of diclofenac sodium:
a) (Control); b) (Diclofenac sodium 0.5 mg/ml)

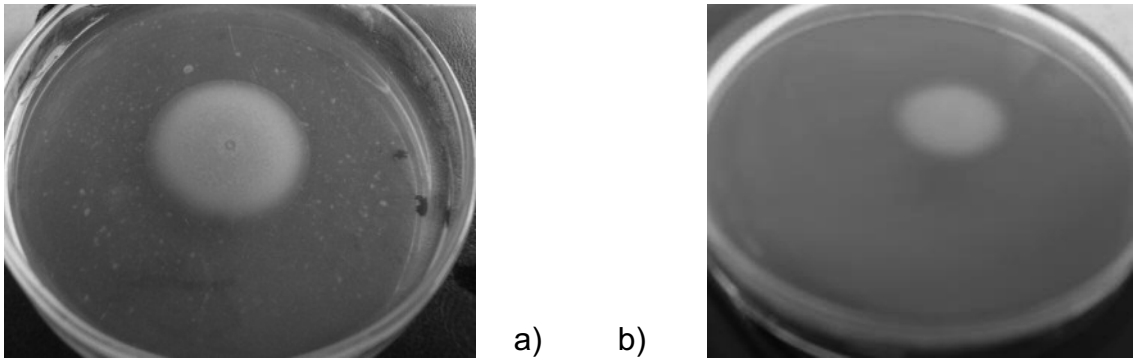

Fig. 2. Inhibition of swimming motility by sub-MICs of diclofenac sodium:
a) (Control); b) (Diclofenac sodium 0.5 mg/ml)

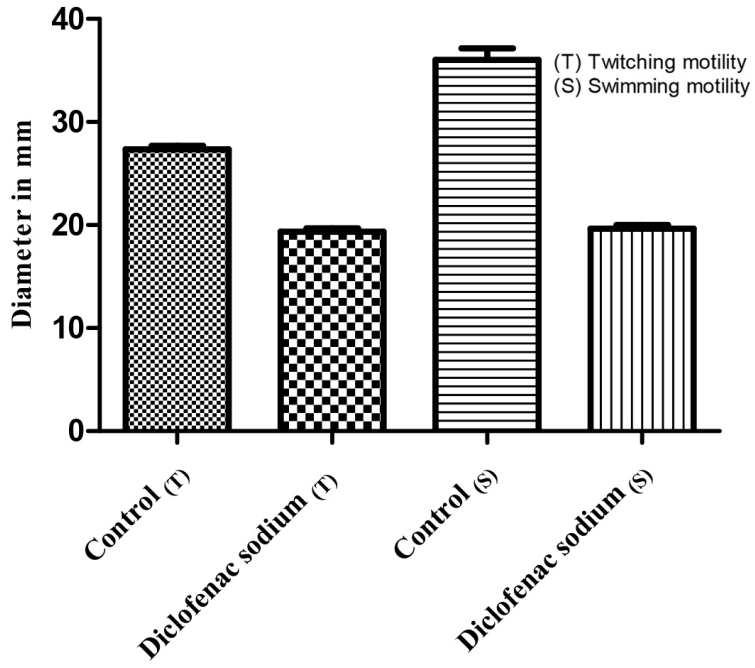

Fig. 3. Inhibition of twitching and swimming motilites by sub-MICs of diclofenac sodium. The results are expressed as mean±SD of three independent experiments.

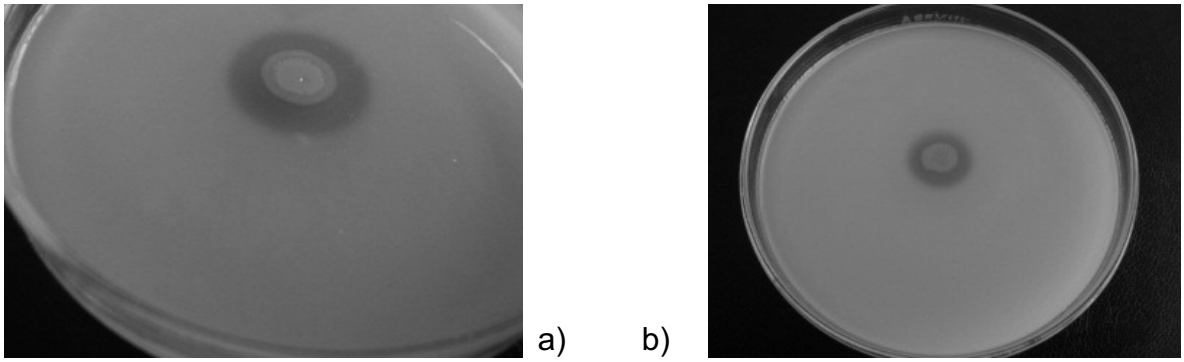

Fig. 4. Inhibition of protease by sub-MICs of diclofenac sodium:
a) (Control): b) (Diclofenac sodium 0.5 mg/ml)

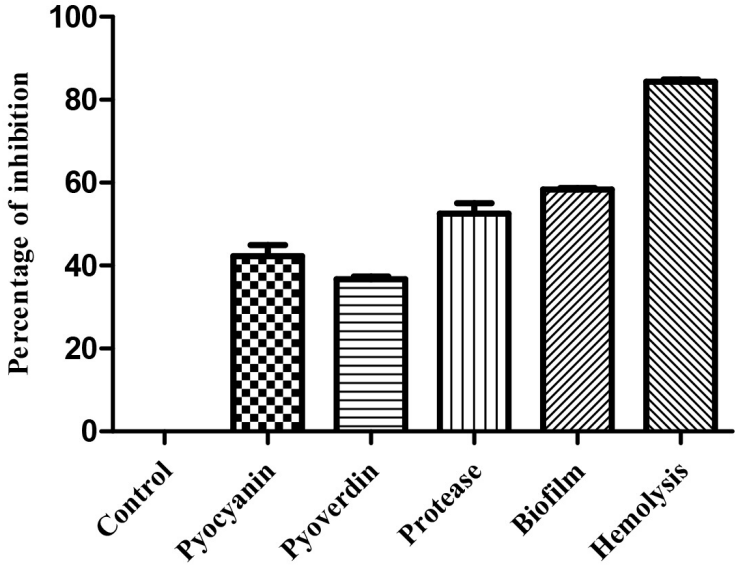

Fig. 5. Inhibition of virulence factors (pyocyanin, pyoverdin, protease, hemolysin and biofilm) by sub-MIC of diclofenac sodium. The results are expressed as mean±SD of three independent experiments

Inhibition of Virulence factors

Significant decrease in the production of protease, haemolysin, pyoverdin, pyocyanin and biofilm formation was observed as compared to the control ($P< 0.05$) as shown in figure 5. Diclofenac sodium inhibited protease by 52.58% (Figures 4 and 5). The inhibition of biofilm by diclofenac sodium was 58.37%. Furthermore, haemolytic activity in the presence of diclofenac sodium was 15.64% or in other words, the haemolytic activity was inhibited by 84.36%.

Diclofenac sodium showed significant inhibitory activity against pyocyanin and Pyoverdin production as compared to the control ($P< 0.05$) (Figures 5, 6). The inhibitory effect of diclofenac sodium against these two virulence factors was less than other factors. It reduced pyocyanin production by 42.32% and pyoverdin by36.72%.

DISCUSSION

Targeting virulence factors is an alternative option to combat the emergence of resistance to antimicrobial agents due to lack of pressure on bacterial growth [24]. The virulence factors of *Pseudomonas aeruginosa* such as protease, haemolysin, pyocyanin and biofilm formation are regulated by quorum sensing genes [25, 26]. As a result, targeting quorum sensing could inhibit the production of *P. aeruginosa* virulence factors.

In this study, the antibacterial activity of diclofenac sodium was determined by determination of MIC. The antibacterial activity of non-steroidal anti-inflammatory agents may be due to interference with bacterial DNA synthesis [27]. Further, 1/4 MICs of diclofenac sodium was used to test the effect on virulence factors without affecting the bac-

terial growth. The biofilm formation capacity of the tested clinical isolate was investigated. The isolate was found to be strong biofilm producer.

Interference with bacterial motility impairs bacterial adhesion and biofilm formation [28]. In this study sub-MIC of diclofenac sodium showed a significant reduction in swimming and twitching motilities and biofilm formation. Diclofenac sodium at sub-MICs showed significant inhibitory activities against pyocyanin, pyoverdin, protease and haemolysin.

The effect of non-steroidal anti-inflammatory agents on virulence of *P. aeruginosa* was previously reported. Salicylic acid inhibited twitching, swimming and swarming motilities and biofilm formation in *P. aeruginosa* [29]. Aspirin was also found to inhibit quorum sensing and virulence factors in *P. aeruginosa* including protease, haemolysin, pyocyanin, twitching and swimming motilities and biofilm formation [30].

Diclofenac sodium was found to significantly decrease colonization of contact lenses pre-colonized with *Pseudomonas aeruginosa* [31]. NSAIDs might inhibit biofilm production by interference with bacterial adhesion, decreasing extracellular polysaccharide and alteration of the properties of the bacterial cell surface [32, 33].

The activity of diclofenac sodium against virulence factors of *P.aeruginosa* may be due to quorum sensing inhibition. Diclofenac sodium could decrease the production of quorum sensing-dependent virulence factors in *P. aeruginosa*. It inhibited swarming motility by 84%, while the pyocyanin inhibition was to a lesser degree [34].

CONCLUSION

Diclofenac sodium may be a useful anti-virulence agent against *P. aeruginosa.* As a result, it is suggested to use diclofenac sodium in the treatment of *P. aeruginosa* infections to avoid the emergence of resistance to antibiotics because there is no stress on bacterial growth.

Conflict of Interest: There is no conflict of interest.

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RELATIVE EXPRESSION OF EFFLUX PUMPS IN MULTI DRUG RESISTANT *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Pseudomonas aeruginosa is known as an important opportunistic pathogen, resistant to a high number of antibiotics. Efflux pumps are one of the main intrinsic antibiotics resistance mechanisms in *P. aeruginosa*. MexAB-OprM, MexCD-OprJ, and MexXY-OprM are the main efflux pumps involved in beta-lactam resistant strains which may cause cross resistance to different antimicrobial classes. The aim of this study was to detect relative gene expression in betalactam-resistant clinical *P. aeruginosa* strains. One hundred fourteen clinical strains of *P. aeruginosa* were identified by phenotypic and genotypic methods. Antibiotic susceptibility testing was conducted according to CLSI guideline. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an efflux pump inhibitor for phenotypic detection of efflux pump mechanism and q-RT PCR was conducted for relative gene expression detection.

The highest rate of resistance was observed against cefotaxime and various relative gene expressions levels were observed in all isolates with positive phenotypic test results.

REZUMAT

Pseudomonas aeruginosa este cunoscut ca un important patogen oportunist, rezistent la un număr mare de antibiotice. Pompele de eflux reprezintă unul dintre principalele mecanisme intrinseci de rezistenţă la antibiotice ale *P. aeruginosa.* MexAB-OprM, MexCD-OprJ şi MexXY-OprM sunt principalele pompe de eflux ale tulpinilor rezistente la beta-lactam, care ar putea sta la baza rezistenţei încrucişate la diferite clase de antimicrobiene. Scopul acestui studiu a fost să se detecteze expresia genetică relativă a izolatelor clinice de *P. aeruginosa* rezistente la beta-lactam. 114 tulpini clinice de *P. aeruginosa* au fost identificate prin metode fenotipice şi genotipice. Testarea sensibilităţii la antibiotice s-a efectuat conform ghidului CLSI. Apoi s-a folosit carbonil cianid 3-clorofenilhidrazona (CCCP) ca inhibitor al pompei de eflux pentru detectarea fenotipică a mecanismului pompei de eflux, iar q-RT PCR s-a efectuat pentru detectarea expresiei genetice relative.

Rata cea mai înaltă de rezistenţă s-a observat faţă de cefotaxim; s-au observat diferite nivele relative de expresie genetică la toate izolatele cu rezultate fenotipice pozitive.

Keywords: Efflux pump, inhibitor, relative expression

INTRODUCTION

Pseudomonas aeruginosa is one of the opportunistic pathogens, especially in immune suppressed patients such as burn ones [1-3]. Involvement of *P. aeruginosa* has been reported in various types of infections, and is the first cause of nosocomial infection in burn patients, in many countries, like Iran [4]. High antibiotic resistance is one of the most important peculiarities in *P. aeruginosa* [1-3]. A frequent innate antibiotic resistance mechanism is related to various efflux pumps in this bacterium [5].

The efflux pumps were identified in 1980 for the first time [4]. Mex efflux pumps group is one of the most prevalent efflux pumps in *P. aeruginosa,*

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confering cross resistance in this gram-negative bacterium [5-9]. Actually, each Mex group of efflux pumps includes three proteins encoded by genes located on the chromosome [5]. Over expression of efflux pump genes caused the rejection of antibiotics outside of the bacterial cell, conducting to an increase of antibiotic MIC which is leading to antimicrobial resistance [10].

According to literature data, i. MexXY-oprM can lead to aminoglycosides, beta-lactams and fluoroquinolones resistance. ii. MexEF-oprN is related to resistance to fluoroquinolones. iii. MexCD-oprJ and MexAB-oprM can lead to beta-lactams and fluoroquinolones resistance [5-7].

Detection of efflux pumps genes expression is useful as it can provide an explanation of cross resistance in *P. aeruginosa* and it may play an important role in this gram-negative bacteria antimicrobial resistance [8-10]. Therefore, the aim of this study was to evaluate gene expression of efflux pumps by quantitative Real-Time PCR (qRT-PCR) in beta-lactam resistant *P. aeruginosa* strains isolated from clinical specimens.

MATERIALS AND METHODS

Bacterial strains and identification

A total of 114 *P. aeruginosa* strains were isolated from two hospitals in Tehran, Iran from April to November 2014. All isolates were transferred to the microbiology laboratory in Iran University of Medical Science and were confirmed by using the biochemical and microbiological tests and the specific primers of *oprI* and *oprL* as described by Vos D. *et al.* [11]. *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used as positive and negative controls, respectively.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar plates, by using Mast antibiotic discs. The disc diffusion method was applied with Cefepime (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Amikacin (30 µg), Ticarcillin (75 µg), Ticarcillin-Clavulanic Acid (75/10 µg), Piperacillin (100 µg), Piperacillin-Tazobactam (100/10 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Tobramycin (10 µg) and Colistin (10 µg) discs and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI 2013) recommendations.

RNA Extraction

The strains were cultured in LB medium (5 mL) and were grown to mid-exponential phase (OD600 = 1.5–2.0). Then, the bacteria ($5*10^8$) were added to 0.5 mL of RNeasy bacteria protect solution (Qiagen, 74104, Germany) to extract RNA according to the supplier instructions. Furthermore, DNA was eliminated using 20U of RQ1 RNAse-free DNAse (Promega, Madison, WI) and resuspended in 50 µL of DEPC-treated water (0.1% v/v) [12].

Efflux pump Detection

To detect efflux pump and to determine MIC, CCCP (C2759 Sigma-Alderich, France) as an efflux pump inhibitor (25 µg/ml) [14] was added to each of Mueller-Hinton agar (MHA) plates containing 0.5 to 1024 µg/ml Cefepime and 0.5 to 256 µg/ml Imipenem (according to CLSI recommendation) as a representative of broad spectrum Cephalosporin and broad spectrum beta-lactam, respectively. The positive criterion for the presence of efflux pump was an at least 4-fold decrease when CCCP was added [13].

cDNA Synthesis

The RNA sample (1 µg) was incubated with 250 ng random hexamer primers (Sigma) and was added to the premix cDNA synthesis kit (BioNEER, Cat. No. K- 2041, Korea). The reaction was performed for 60 seconds at 15 °C, 60 min at 55 °C and then at 95 °C for 5 min.

Real-time qPCR Reaction

The qPCR primers recommended by other authors were used as shown in Table 1. A Rotor Gene RT-PCR machine (Corbett Research, Sydney, Australia; Model RG3000, software version 6) was used for the duplicated PCR reactions with the QuantiT ect SYBR Green RT-PCR Kit (Qiagen, Cat. No. 204243). After 12 min activation of the modified Taq polymerase at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at each gene annealing temperature (Table 1) and 30 s at 72 °C were performed. Then, the ΔCT values were used for data analysis [14].

RESULTS

All isolates have been confirmed by biochemical and molecular tests. The highest resistance rate of 86% was observed against Cefotaxime. No resistance was detected against Colistin. The percentage of resistance to all different tested antibiotics is shown in Fig. 1. The MIC variation (decrease)

Table 1. **Real-Time PCR primers**

Primer	Sequence (5'→3')	PCR product (bp)	References
MexA F	CGACCAGGCCGTGAGCAAGCAGC	316	12
MexA R	GGAGACCTTCGCCGCGTTGTCGC		
MexC F	GTACCGGCGTCATGCAGGGTTC	164	12
MexC R	TTACTGTTGCGGCGCAGGTGACT		
MexX F	TGAAGGCGGCCCTGGACATCAGC	326	12
MexX R	GATCTGCTCGACGCGGGTCAGCG		
MexEF MexER	CCAGGACCAGCACGAACTTCTTGC CGACAACGCCAAGGGCGAGTTCACC	114	12
16sRNA F	AACGGACGACCATCTTTGAGTATT	151	15
16sRNA R	CAGTTGTTCCATTTCACGCATT		

against beta-lactam antibiotics (Imipenem and Cefepime), Gentamicin and Ciprofloxacin in the presence of CCCP is presented in Table 2. The decrease of MIC in the presence of CCCP was between 4 fold and more than 16 fold (Table 2). The relative expression of MexX, MexA and MexC genes was increased up to 256, 8 and 8 fold respectively in strains with decrease of MIC in the presence of CCCP. The effect of efflux pumps on antibiotics resistance was observed in all strains with decreased MIC in the presence of CCCP.

The relationship between decrease of tested antibiotics MIC in the presence of CCCP and efflux pump genes expression is shown in Table 3.

DISCUSSION

Antibiotic resistance is one of the main and challengeable characters in *P. aeruginosa* [3, 16, 17]. In addition to acquisition of antibiotic resistance genes, intrinsic mechanisms such as over expression of efflux pumps play an important role in *P. aeruginosa* [5]. MexA, MexC and MexX are members of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps classes, respectively, that are involved in beta-lactam resistance in these gram-negative bacteria [5- 6]. The results of this study indicate that all isolates that prove to be beta-lactam resistant by phenotypic methods show various degrees of over expression in MexA, MexC and MexX genes. The results of our study conducted in Iran, in 2014,

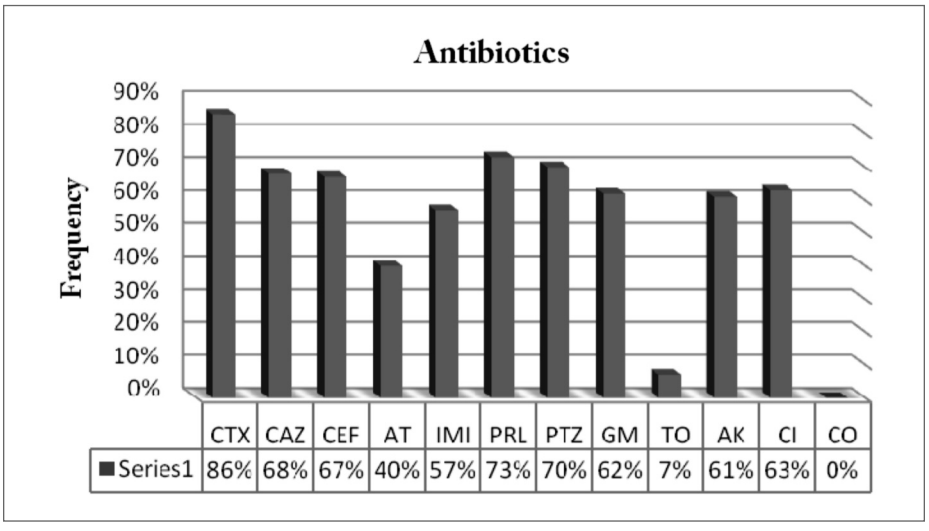

Fig. 1. - Percentage of antibiotic resistance

CTX: Cefotaxime, CAZ: Ceftazidime, CEF: Cefepime, AT: Aztreonam, IMI: Imipenem, PRL: Piperacillin, PTZ: Piperacillin-Tazobactam, GM: Gentamicin, TO: Tobramycin, AK: Amikacin, CI: Ciprofloxacin, CO: Colistin

Table 2. **Variation (decrease) of cefepime and imipenem MIC with CCCP**

	Variation (decrease) of MIC in the presence of CCCP	Frequency of strains with decrease of MIC (%)
Cefepime	4 - ≥8 fold	7
Imipenem	≥8 fold	9
Cefepime & imipenem	4 - ≥8	7

Table 3. **The relationship between decrease of beta-lactam MIC in the presence of CCCP and increase of efflux pump relative genes expression**

Antibiotics	Decrease of MIC in the presence of CCCP	MexA gene expression increase	MexC gene expression increase	MexX gene expression increase
Imipenem	4 - ≥8	4-8 fold	2-8 fold	4- 128 fold
Cefepime	≥8	4 fold	4 fold	32- 256 fold
Imipenem and Cefepime	4 - ≥8	4 fold	8 fold	4-256 fold

showed over expression of MexA and MexX in the tested isolates [18]. Sacha *et al*. indicated the over expression of MexAB-OprM pump in their isolates [19]. Also, Gonge *et al*. showed that the high expression of the MexA-MexB-OprM efflux pump could increase antibacterial resistance and promote infection [20]. The results of another study conducted in Japan demonstrated the effect of MexCD-OprJ over expression in penem resistant *P. aeruginosa*. In that study, MexAB-OprM was the most important among the three efflux systems [21]. In this respect, our results showed over expression of MexA, MexC and MexX genes in some of MDR strains. It is notable that some of our tested isolates that were beta-lactam resistant showing at least 4 fold MIC decrease in the presence of CCCP remain in the intermediate MIC range (16%). This indicates the important role of efflux pumps genes over expression in antibiotic resistance characterization of these *P. aeruginosa* strains. It is possible that other antibiotic resistance mechanisms participate, which could explain why these resistant strains remain in intermediate range of MIC after addition of CCCP as an efflux pumps inhibitor. According to our results and previous studies [8, 9] over expression of MexX is able to cause cross resistance between three important antibiotic families, including beta-lactams, aminoglycosides and fluoroquinolones. Similarly, MexA and MexC may cause cross resistance between beta-lactams and fluoroquinolones. Cross resistance in *Pseudomonas aeruginosa* by over expression of efflux pumps may be a therapeutic challenge and can increase the rate of morbidity and mortality, especially in burn patients. So, introduction of efflux pumps inhibitors of clinical usage may be very useful for treating infections caused by these types of bacteria.

Acknowledgements

This study was supported by a grant (M/T 92-01-134-21562) from Iran University of Medical Sciences, Tehran, Iran.

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STUDY TO ESTABLISH THE ACCEPTANCE RANGE FOR PEROXYL RADICALS SCAVENGER CAPACITY OF *NATURAL SOD*

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ABSTRACT

In the context of an emerging market of food supplements, the proven quality of the antioxidant products should be the main criteria for using them. The production process has to be carefully controlled and complementary tests are needed to demonstrate the correspondence between real and declared properties of final product. Using well characterized compounds with proven antioxidant activity in biological systems as reference brings a plus of rigorously to the testing protocol. The aim of this study was to determine the acceptance range for the antioxidant (peroxyl radicals scavenger) capacity of "Natural SOD" by using for comparison ascorbic acid (vitamin C). The established acceptance range complete our previous results concerning the antioxidant capacity of Natural SOD using validated ORAC method and creates premises for supplementary checking of the batches in the current production and improving the product quality

REZUMAT

În contextul unei pieţe a suplimentelor nutritive în continuă dezvoltare, calitatea dovedită a produselor antioxidante ar trebui să fie principalul criteriu pentru alegerea şi utilizarea acestora. Procesul de producţie trebuie să fie atent controlat şi sunt necesare teste complementare pentru a demonstra corespondenţa dintre proprietăţile declarate şi cele reale ale produsului final. Utilizarea ca referinţă a compuşilor bine caracterizaţi, cu activitate antioxidantă deja dovedită în sistemele biologice, aduce un plus de rigurozitate protocolului de testare. Scopul acestui studiu a fost determinarea intervalului de acceptabilitate pentru capacitatea antioxidantă (de epurare a radicalilor peroxil) a produsului SOD Natural, comparativ cu acidul ascorbic (vitamina C). Stabilirea intervalului de acceptabilitate completează rezultatele anterioare privind capacitatea antioxidantă a produsului SOD Natural, pe care le-am obţinut utilizând metoda validată ORAC şi creează premisele pentru verificări suplimentare ale loturilor din producţia curentă şi îmbunătăţirea calităţii produsului.

Keywords: Natural SOD, green barley, antioxidant, ascorbic acid, peroxyl radicals scavenger, ORAC

INTRODUCTION

The antioxidants market (with large diversity) is one of the more active markets, either as a classic commerce (drugstores, pharmacies, super/hyper markets or as an online commerce (where the non-authorized commerce is dominant). Similar to classical drugs, using food supplements implies risks and side effects. Furthermore, it should be taken into account that acquiring food supplements is generally made without medical prescription; besides, the marketing strategy of food supplement producers is sometimes aggressive, that also has the effect of "self-prescription" for these products.

The excessive consumption of a food supplement can lead to exceeding the limit below which a certain compound existing in the product has beneficial effects. Furthermore, the insufficient chemical and biochemical characterization of plant extracts frequently used in food supplements formulation, can lead to potentially toxic compounds intake. This is the cause for which more studies are necessary, aiming to compare a certain effect of a food supplement with the same effect of a standardized compound, for which its "beneficial" limits have already been established and validated.

The economic impact of antioxidants is generally based on the biological reality, meaning that for a proper functionality of the organism, a balance between pro-oxidant processes and antioxidant protection is absolutely necessary (Fig. 1).

Pro-oxidative processes are essential for life of aerobic organisms which derived their energy from the reduction of oxygen. These bio-chemical reactions are accompanied by reactive oxygen and nitro-

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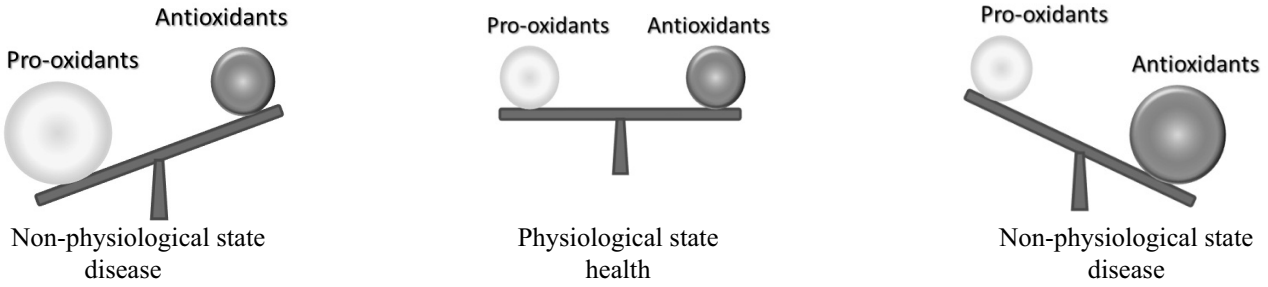

Fig. 1. Changes in pro-oxidants/antioxidants ratio could promote a non-physiological state and disease

gen species (ROS and RNS respectively) generation [1]. The ROS and RNS of particular importance for the biologic systems include: hydroxyl radicals (•HO), superoxide anions ($O2^{-}$•), peroxyl radicals (R-OO•), nitric oxide (NO) and peroxynitrite ($ONOO^{-}$) [2, 3, 4].

Of these, peroxyl radicals play a special role; their excessive production is involved in many pathologies such as neurodegeneration, atherosclerosis, inflammatory diseases [5, 6]. Peroxyl radicals derive from aliphatic radicals through a very fast uptake of molecular oxygen [7] and are involved especially in protein oxidation and subsequent alteration of cell signaling, enzyme activity, thermostability and proteolysis susceptibility [8].

Having a half-life of 0.5-7s and being able to react far from their site of generation, peroxyl radicals are key players in the propagation step of lipid peroxidation. They are converted through a cyclisation reaction to endoperoxides which lead to a wide range of compounds, including malondialdehyde (MDA), a powerful mutagenic compound [9, 10, 11, 12].

Antioxidants act by various both enzymatic and non-enzymatic mechanisms, targeting either the inhibition of ROS production, or neutralization/disposal of already produced radicals. Non-enzymatic antioxidants (e.g. glutathion (GSH), vitamins, phenolic compounds) play a buffering role, neutralizing the already produced free radicals. Enzymatic antioxidants (e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) complete the defence system [8, 13].

A deficit of ROS could be associated with a poor defence against infections and impaired metabolism of toxins [14]. On the other hand, oxidative stress (results of excessive ROS production and inability of antioxidant systems to neutralize these ROS cellular effects) contributes to progression of all diseases with inflammatory component [13, 15, 16, 17, 18, 19, 20, 21, 22, 23]. In pathological conditions, inhibiting the excessive production of ROS and/or scavenging of already produced ROS are very important processes. This is why antioxidants, as correctly formulated bioactive compounds, are necessary to accomplish all the requirements defining the therapeutic success, but only if they are administered to patients with specific necessities, in corresponding amounts, for corresponding durations, in association with drugs recommended in therapeutic protocols.

Many plants contain important amounts of antioxidants (vitamins, carotenoids, flavonoids) that are able to scavenge the excess of free radicals in the human body, can replace endogen antioxidants ineffectiveness in normal cells and slow-down the processes of oxidative destruction. The major advantages of using antioxidants of vegetal origin are their efficacy, low incidence for side-effects and their reduced costs.

Numerous studies demonstrated that green barley is one of the most balanced sources of nutrients for the human body and green barley extract obtained only from young plants (max. 20-25 cm high) has a purifying effect, helping to eliminate many toxins which could potentiate and trigger various pathological processes when accumulate [24, 25, 26, 27, 28, 29, 30, 31, 32, 33].

Natural SOD is a food supplement patented and commercialized by "Cantacuzino" National Research Institute, using only plants of its own crops, cultivated in ecologic conditions, harvested when the plants had a maximum high of 20-25 cm and treated according to a validated technologic process in order to obtain a pure and safe final product. Our previous studies [34] demonstrated the efficacy of *Natural SOD* as antioxidant; it is a good free oxygen radicals (FOR) scavenger, based on its content of micromolecular substances having enzymatic activity (SOD-like, peroxidase-like) and also other scavenger compounds for peroxyl radicals.

Testing for the antioxidant capacity (peroxyl radicals scavenging) of *Natural SOD* product has

several advantages: ORAC is a precise method (the working protocol is already validated by our research group), relatively cheap and allows a better characterization of the product. Comparing to a known antioxidant (other than Trolox which is used for the standard curve) increases the stringency of the samples and allows the detection of any variations concerning the quality of the raw material and/or of the technologic process, which are reflected in the quality of the final product.

Ascorbic acid is one of the most important vitamins with antioxidant properties, with an important role in immunity regulation and in infections prevention. At international level, the recommended daily dose (RDD) of ascorbic acid is 75-90 mg/day for adults (also depending on age and sex). However, in 2002, nutrition researchers have claimed that the dose of 90 mg/day is too small and it should be raised to 200 mg/day. To obtain the optimal antioxidant effect, some specialists recommend a treatment with 200-1000 mg/day ascorbic acid for a period of 1-12 months [35].

The aim of this study was to determine the acceptance range by using for comparison a known and well characterized antioxidant which brings a plus of rigorously to the testing method and contributes to create premises for improving the product quality.

MATERIALS AND METHODS

Experimental design

For the purpose of achieving our objectives, the following steps (Fig. 2) have been carried out:

- Measuring the antioxidant capacity of ascorbic acid (known antioxidant compound) by ORAC method – four known concentrations of ascorbic acid have been tested (6.25 µM, 12.5 µM, 25 µM, 50 µM).
- By using the ORAC method, the antioxidant capacity of 20 samples of *Natural SOD* from 20 different batches (2012-2014) within the period of validity, has been measured, aiming to establish the proven acceptance range.
- A preliminary study, with the purpose to validate the acceptability range by using the ORAC method for testing 10 samples from 10 different batches of *Natural SOD* produced in 2014, has been performed.

ORAC (Oxygen Radical Absorbance Capacity) method)

ORAC *in vitro* method is used to measure the scavenger activity of a potential antioxidant compound against peroxyl radicals produced through thermal decomposition of 2,2'-azobis (2-amidino propane) dihydrochloride (AAPH). The process is revealed by changes in fluorescence intensity of fluorescein molecules which are degraded by peroxyl radicals. Reducing the amount of oxidizing radicals, an antioxidant compound is able to delay the degradation of fluorescein. Trolox - a synthetic analogue of Vitamin E – is used as standard antioxidant [36, 37, 38].

ORAC measurements were performed with respect to the previously published experimental protocol [34], briefly presented in Fig. 3.

All chemicals were purchased from Fluka. The sample corresponding values have been calculated in relation to the Trolox standard curve and were expressed as Trolox equivalents/liter (TE/l); 1 TE = 1 mM Trolox.

RESULTS AND DISCUSSION

The results obtained after measuring the antioxidant capacity of ascorbic acid (well-known and well characterized antioxidant) by using the ORAC method, are presented in Figure 4. Individual values represent the average of the corresponding triplicates

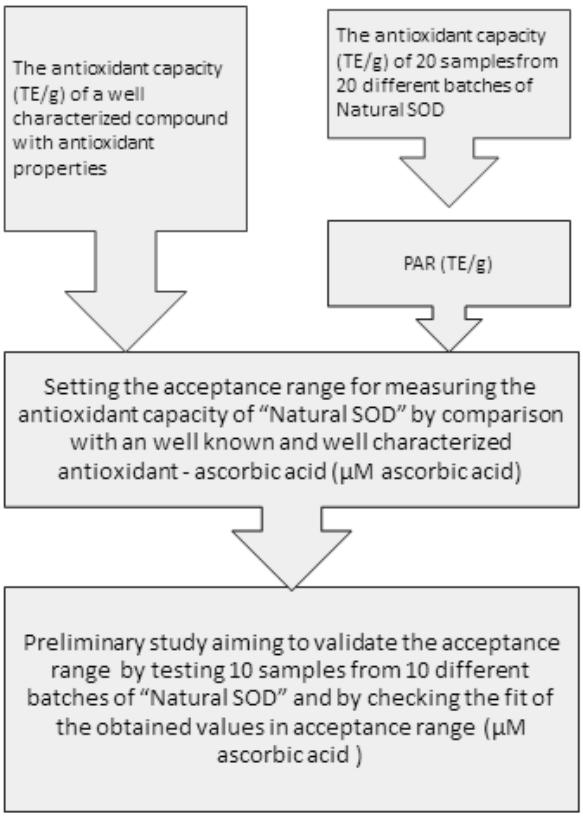

Fig. 2. Schedule of the experimental design for establishing the acceptability range of *Natural SOD* using ORAC method

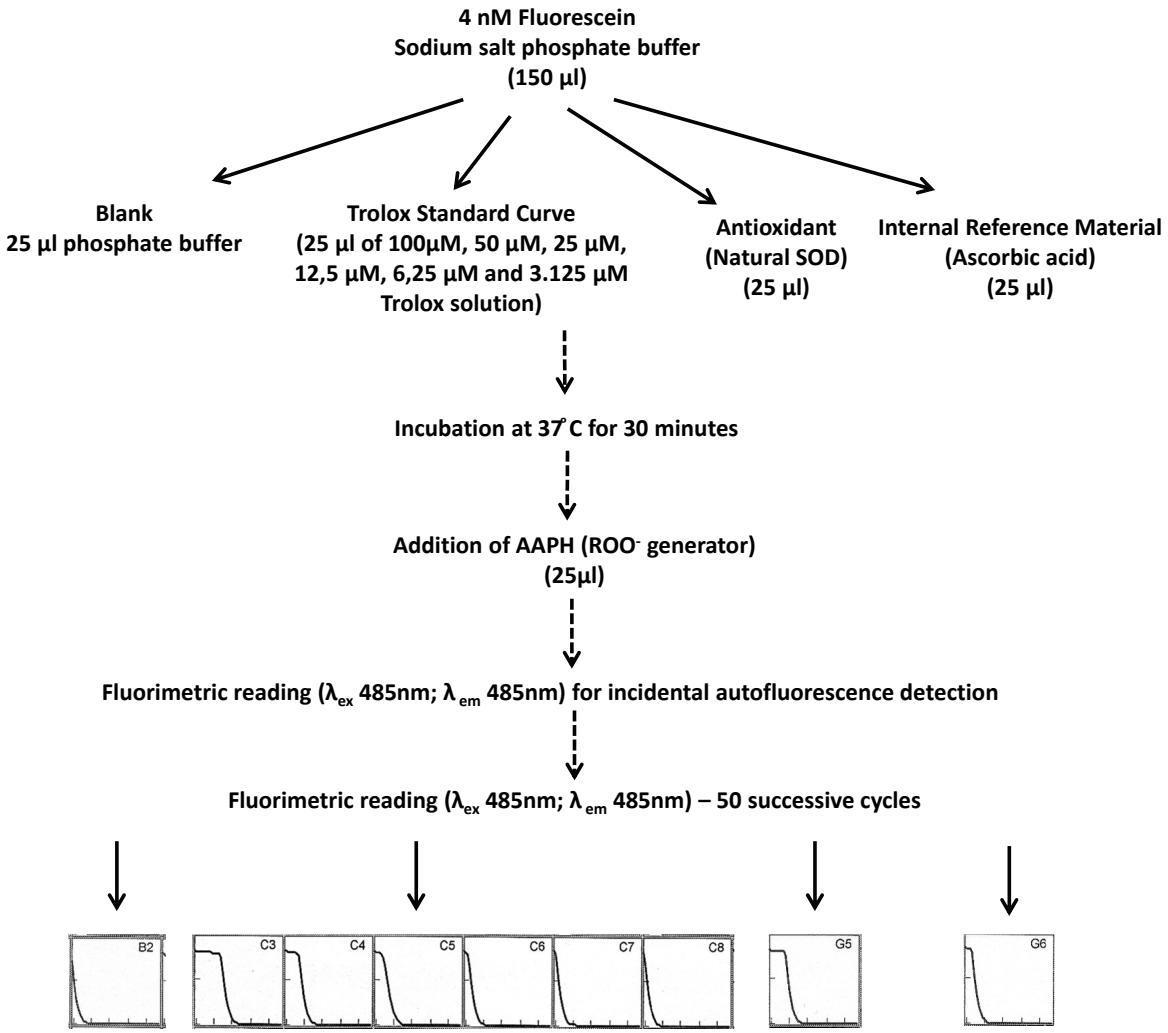

Fig. 3. ORAC experimental protocol for measuring the peroxyl scavenger capacity of *Natural SOD*

for each tested concentration of ascorbic acid. The variation coefficient corresponding to each value was <10%. In order to test the compliance with Beer-Lambert law for the concentrations chosen range, the correlation coefficient R^2 was calculated. The obtained value R^2= 0.993 (>0.95) reflects the correct choice of ascorbic acid as a reference compound for testing the antioxidant capacity by ORAC method.

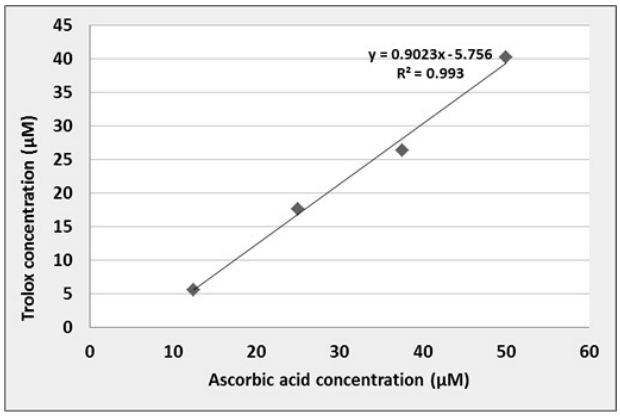

Fig. 4. The antioxidant capacity of ascorbic acid (6.25 – 50µM) measured by ORAC method

ORAC values obtained for 20 samples (within the period of validity) from 20 different batches of *Natural SOD* (2012-2014) are presented in Fig. 5.

Individual values represent the average of the corresponding triplicates for each of the tested batches. The variation coefficient corresponding to each batch was <10%. Average and standard deviation have been calculated for the string of values corresponding to the 20 tested batches. The acceptance range (minimum and maximum accepted limits) for measuring the antioxidant capacity of *Natural SOD* product by ORAC validated method, compared with a known antioxidant (ascorbic acid) has been established as being x+/-2s, where x = average, and s = standard deviation.

Setting the acceptance range allows the use of a control charts for verifying the antioxidant capacity

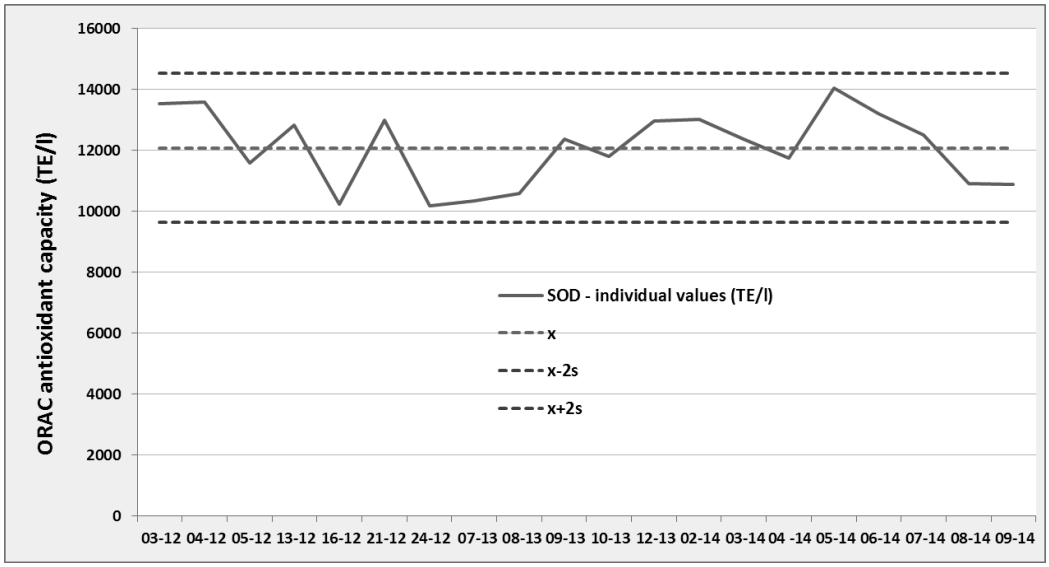

Fig. 5. ORAC values obtained for 20 samples (within the validity period) from 20 different batches of *Natural SOD* (2012-2014 production)

(tested by ORAC method) of the batches in the current production. Corresponding values for the ten tested batches of *Natural SOD* (other than those used for determination of the acceptance range) fall within the upper limit (x+2s) and the lower limit (x-2s) (Fig. 6). Moreover, no systematic deviations (more than three successive values of one or the other side of the corresponding average line) were detected, demonstrating the appropriate testing of the product and the maintaining of its quality from one batch to another.

To compare the antioxidant capacity of *Natural SOD* batches with the antioxidant capacity of ascorbic acid, the obtained values above have been converted into μM ascorbic acid and then into mg/l ascorbic acid. Data are presented in Fig. 7.

Thus, the acceptance range becomes 18684.45 – 28149.11 mg ascorbic acid/l (18.684 – 28.149 g/l). Taking into account the fact that an ampoule of *Natural SOD* contains 5 ml product, by calculations we conclude that an ampoule of *Natural SOD* has the antioxidant (peroxyl radical scavenger) capacity of 93.422 mg ascorbic acid. The daily dose recommended by Mayo Clinic [35] for obtaining an antioxidant effect is 200-1000 mg/day. These calculations argue that the recommended daily intake (RDI) of *Natural SOD* (2 ampoules/day) is within the ascorbic acid range recommended for

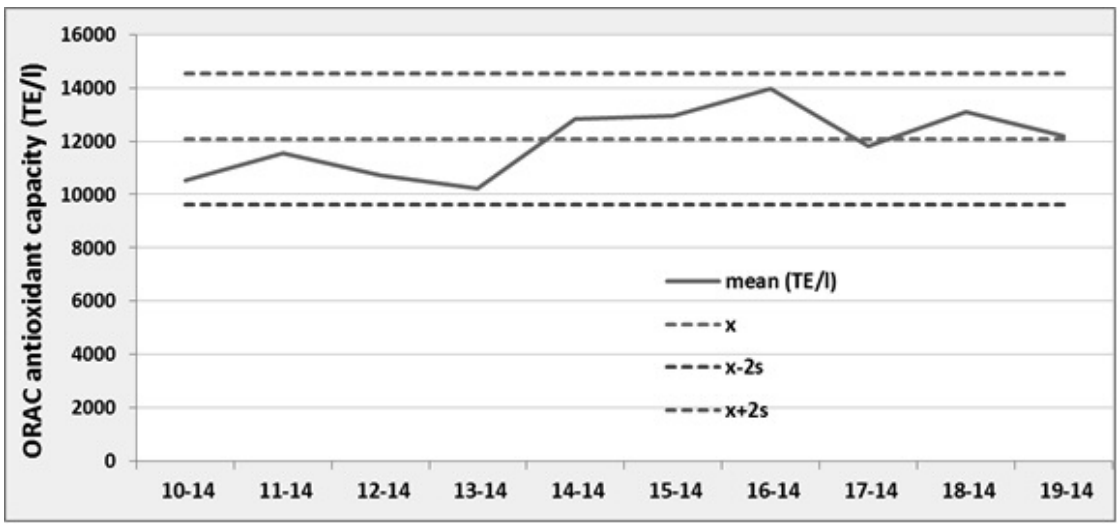

Fig. 6. ORAC values corresponding to 10 tested *Natural SOD* batches produced in 2014

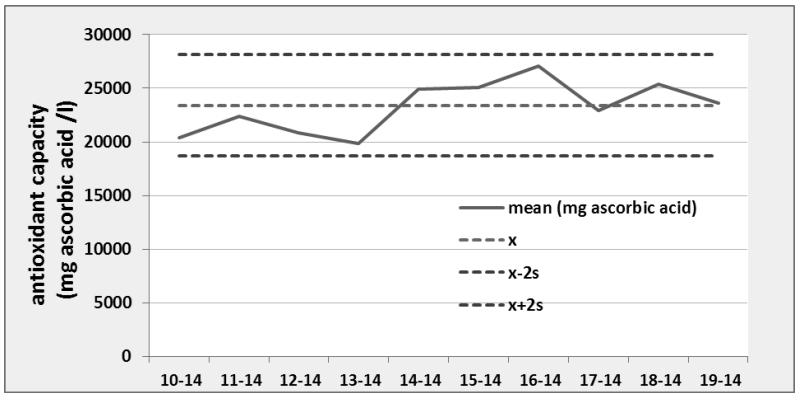

Fig. 7. The acceptance range (mg acid ascorbic/l) and the antioxidant capacity of 10 tested *Natural SOD* batches produced in 2014

obtaining an antioxidant effect (200-1000 mg/day) but this affirmation needs to be confirmed by experimental data. On the other hand, these results draw attention to the fact that, like other food supplements, *Natural SOD* could not replicate all the needed nutrients and should not substitute a balanced diet.

Moreover, containing a mixture of compounds with antioxidant capacity, the beneficial effect of *Natural SOD* is higher compared with the effect of individual compounds, which displayed antioxidant or pro-oxidant activity [39, 40]. Excessive intake of a certain antioxidant could induce dental decalcification, blood hemolysis, tumor cell proliferation, kidney stones, increased risk of cardiovascular disease etc, in the context of interference of antioxidants with essential defense mechanisms (e.g. apoptosis), detoxification mechanisms, impaired absorption of other essential compounds, competition for substrate binding and damage to essential lipids [40, 41].

The permanent interactions between different antioxidants in biological systems make the reduction of oxidative stress and his pathological consequences to require the addition of a complex of antioxidants able to act by multiple mechanisms for restoring the equilibrium (physiologic oxidative status).

Our results reflect the capacity of *Natural SOD* to scavenge peroxyl radicals, without assigning this property to a particular class of compounds. Elucidation of this aspect constitutes an interesting research topic, aimed to develop and to improve the quality of *Natural SOD*. The hypothesis that the peroxyl scavenger capacity of *Natural SOD* contributes to the antiinflammatory properties of the product, should be also investigated.

CONCLUSIONS

Using ORAC method, we established the acceptance range for the antioxidant (peroxyl radical scavenger) capacity of *Natural SOD*, compared with ascorbic acid, a well characterized antioxidant. This range is: 18684.45 – 28149.11 mg ascorbic acid/l (18.684 – 28.149 g/l).

Establishment of the acceptance range enables using a control chart in order to check by ORAC method the antioxidant capacity corresponding to the batches in the current production, with a plus of rigorously and specificity by comparing with the already used enzymatic methods (SOD-like and Peroxidase-like activities).

Moreover, a better characterization of *Natural SOD* using validated methods creates premises for improving the product quality.

Acknowledgements

This study was supported by the National Research Program, Grant No. PN 09220203/2014. We are grateful to Stefania Dascalu for technical assistance.

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IMMUNOLOGICAL IMPLICATIONS IN MAMMALIAN SEMI-ALLOGENEIC PREGNANCY

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ABSTRACT

The mammalian conceptus is a semi-allograft. The maternal tolerance towards semi-allograft is primed by a battery of cytokines and resident decidual cells. Invasion of embryonic trophoblast, decidual vascular remodeling and unresponsive incipient 'Triple-negative' ($CD3^-$, $CD4^-$ and $CD8^-$) T-cells of foetus promote normal pregnancy. On the other hand, the maternal cytokine profile shift towards Th1 response is shown to enhance the risk during pregnancy. The periodical examination of maternal serum levels of Th1 and Th2 response mediated cytokines constitutes prognostic biomarkers to initiate therapeutic regimens.

REZUMAT

La mamifere, embrionul este o semi-alografă. Toleranţa maternă faţă de semi-alografă este amorsată de o baterie de citokine şi celule deciduale locale**.** Invazia trofoblastului embrionic, remodelarea vasculară deciduală şi celulele T „triplu negative" neresponsive şi incipiente ($CD3^-$, $CD4^-$ and $CD8^-$) ale foetusului sunt promotoare ale unei sarcini normale. Pe de altă parte, profilul matern de citokine modificat spre răspuns Th1 pare să sporească riscul în timpul sarcinii. Investigarea periodică a nivelelor serice materne de citokine Th1 şi Th2 mediate constituie biomarkeri de prognostic pentru iniţierea regimurilor terapeutice.

Keywords: Semi-allograft, pregnancy, immune cells, cytokines, biomarkers

INTRODUCTION

The zygote, implants in parous woman that further grows as foetus, is found to be unique being tolerated by the maternal immune system. Whether the foetus is immune-privileged or the parous woman is immunosuppressive or both cleverly adopt a transient immune compromization or the feto-maternal interface protects the foetus, need to be understood in a wider perspective of the evolution of viviparity. Importantly, targeting 'non-self' is an attribute of adaptive immune system [1]. In contrast, viviparous organisms are tolerating 'semi-self' conceptus. The same must have been an evolutionary adaptation for the race to continue [2]. Though there is a wide array of immune parameters namely hormones [3], HLA [4], cytokines [5], receptors [6], ligands [7], defense cells [8, 9], antigen presentation [10, 11] and antibodies participation [12] in the pregnant women, yet she maintains a perfect balance in accepting foetus despite being characterized by having desperate paternal antigens. The interplay among immune activation, biased immunity, immune suppression and immune regulation needs to be looked to unfold the disorders of pregnancy.

In the present mini review, it is envisaged to elaborate on the pregnancy-induced immune alterations and the potential biomarkers to avert risks during pregnancy.

Immune components of primiparous women: Rejection of an allograft is one of the inbuilt mechanisms of cell mediated immunity [1]; primarily the allograft is inevitably characterized by individual specific peptides named MHC Class I and II. Between these two groups of antigens, MHC class I is expressed on all nucleated somatic cells which must have been the primary cause to abort fetuses with somatic cell nuclear transfer shown by Davies [2]. The foetus being a semi-allograft is sharing only one half of the MHC expression in common with the maternal tissues.

Immune cells: The foetal development through the progressive pregnancy during penultimate second and ultimate third trimesters coincides with an increase in acquiring immune privilege by the foetus and in the process takes the lead by recruiting a number of decidual immune cells through the interaction

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of stromal cells and a battery of cytokines. Among the immune cells, uterine natural killer cells (uNK) [13], T-cells [14, 15], dendritic cells and macrophages [16,17] are the significant contributors due to their mucosal infiltration during luteal phase and also in the first trimester. Both leukocytes and lymphocytes are shown to aggregate in the uterus [18]. Soon after implantation, predominantly NK cells and macrophages appear at the site of invasion [19]. The prevailing hormonal environment in uterine endometrium influences immune cells to bind to the resident decidual stromal cells utilizing the intercellular adhesive molecules [20].

NK cells are endowed with differential potential to perform. Peripheral NK cells are of two subsets namely dim and bright due to the difference in the density in the expression of CD56 surface receptors. Uniquely, uNK cells are characterized by having surface receptors CD 56^{bright}, $CD16^{-}$ and $cyCD3^{+}$ and they are found to be immunoregulatory involving the cytokine production [21]. In contrast, peripheral NK cells are dim ($CD56^{+dim}$ $CD16^{+}$, $cyCD3^{-}$) and they are highly cytolytic. Normally, NK cells exhibit discrete specificity through their conventional activating and inhibitory receptors and lyse the worn out /abnormal/foreign cells that either least or fail to express 'self' HLA I. Further, this HLA I group is classified as (a) classical which consists of HLA-A, HLA-B, and HLA-C and among them the first two are highly polymorphic and (b) non-classical antigens comprising of HLA-E, HLA-F and HLA-G with least polymorphism. It is shown that both these classical and non-classical HLA molecules interact in decidua and participate in spiral artery remodeling [22] to provide nutrition to the growing foetus. The polymorphic classical HLA molecules of the semi-allograft especially HLA-A and HLA-B are least expressed, whereas the less polymorphic HLA-C and non-classical HLA-G are more pronounced in the trophoblast which must have been a possible motivation from the foetus to prompt maternal immune tolerance and also effectively inhibit maternal immune cells. Furthermore, it has been reported that there is no evidence that NK cells are aggressive during pregnancy [21]; instead they facilitate in angiogenesis in the interface of uterus and trophoblast. Reproductive hormone, progesterone also alters cytokine balance and inhibits foetal tissue lysis mediated by NK and CD8 T cells. In addition, during pregnancy maternal immune system gears more towards humoral immunity and keeps the cell mediated immunity under check to avert foetal tissue damage. Therefore, uNK cells play a profound role in remodeling tissue at the site of implantation and building the placenta.

Macrophages do play active part in both innate and adaptive immunity due to their potential role in processing pathogens and tissue remodeling. Immunomodulatory roles of deciduous macrophages and their involvement in normal and pathological pregnancies have been reviewed by Takeshi Nagamatsu [23] and elucidated that decidual macrophages behave as an immunosuppressive phenotype by producing IL-10 and indoleamine 2, 3 - dioxygenase activity to promote maternal immune tolerance and decidual vascular remodeling during pregnancy. During implantation, decidual tissue remodeling and vascularisation are imperative and the resultant apoptotic cells are to be scavenged by decidual macrophages. Otherwise, a few of embryonic apoptotic cells possibly dwell in the maternal circulation and elicit immune response against the semi-allogenic foetus. Further, it is also shown that the malfunctions due to the aberrant decidual macrophages resulted into the abnormal pregnancies such as pre-eclampsia and preterm delivery [17].

In addition to NK cells and macrophages, T-regulatory cells also contribute toward building maternal tolerance. It is reported that the maternal immune environment during pregnancy induces the generation of well defined T regulatory cells and their subsets namely $CD4^{+}$, $CD25^{+}$ and $FoxP3^{+}$ to prevent rejection of the foetus [24].

Cytokines: Cell to cell communication is invariably facilitated by small molecules secreted into the interstitial fluids. They are variously named as cytokines/lymphokines/interleukins due to their specific involvement. Interleukins are having multiple and redundant functions in promoting the elicitation of immune response [25]. Progesterone is an effective inducer of the production of Th2-type cytokines, LIF (leukemia inhibitory factor) and M-CSF (macrophage colony stimulating factor). *In vitro* studies have shown that the interaction between the trophoblast and uNK cells induced the production of several cytokines and growth factors related to placental development including the following: 1) tumour necrosis factor (TNF-α); 2) interferon gamma (IFN-γ); 3) granulocyte macrophage colony-stimulating factor (GM-CSF); and 4) macrophage inhibitory factor (MIF) [25-27]. In furtherance of the maternal hormonal influence, the decidua, trophoblast and the embryo take the lead in the regula-

tion of cytokine interplay. Ponzio *et al*., [26] administered murine IL-2 to pregnant mice during midgestation, analyzed their offspring (IL-2 pups) and compared the offspring of pregnant mice injected with placebo, phosphate buffered saline (PBS pups). They also reported that considerable levels of IL-2 were noticed in amniotic fluid and tissues of embryos, confirming that the injected IL-2 crossed the placenta and entered fetuses. Further, IL-2 pups witnessed the accelerated T cell development, with an orientation toward TH1 cell differentiation. The growth factors produced by endometrial NK cells were found to inhibit TNF-β and IFN-γ and the participation of both Th2 and Th1 cells were reported in pregnancy [27]. *In vitro* behavior of PBMCs obtained from normal and recurrent spontaneous abortion (RSA) women co-cultured with PHA (phytohemagglutinin) was evaluated in relation to their secretions [28]. Interestingly, PBMCs of normal first trimester pregnant women were reported to secrete high concentrations of Th2 mediated interleukins in culture medium namely IL-4, IL-6 and IL-10, whereas IL-2, IFN-γ, TNF-α and TNF-β were found higher in the cultures of PBMCs obtained from the RSA group suggesting that there exists Th2 bias in normal pregnancy as against Th1 bias. While the Th2-type cytokines (IL-4, IL-5, IL-10) were also reported to inhibit the Th1 responses so as to promote allograft tolerance and therefore improve fetal survival [28]. In addition, IL-5 was found to stimulate uNK cells whose role in the augmentation of trophoblastic and cytotrophoblastic invasion were well acknowledged [5,9]. Piccinni [29] reported that T cells' LIF, M-CSF, IL-4 and IL-10 production at the fetomaternal interface contribute to the maintenance of pregnancy. In an experimental design to evaluate the lapses due to Th1 interleukins during pregnancy, it is shown in mouse models that an increase in maternal levels of IL-2 during pregnancy induced long-lasting vulnerability to neurobehavioural abnormalities associated with autism among offspring [26]. The most interesting contribution from the trophoblast is IL-15 which mediates cell to cell interaction between uNK cells and decidual cells to develop decidual integrity and spiral artery at the site of implantation. Therefore, Th2 mediated immune surveillance predominates not only during pregnancy but also influences the offspring for a better survival.

Trophoblast as an incipient mediator: The zygote through multiple cleavages reaches a stage namely blatocyst that freely floats in uterus and, develops communion and adhesive interaction with the uterine endometrium. Importantly, the outer layer (outer mass of cells) of blastocyst namely trophoblast initiates for the invasion as the first step for implantation, primarily to secure nutrition to the growing inner mass of cells. To ensure the same, the blastocyst in the uterus relays on the unique specialized cell type known as functional trophoblast derived from the trophoecotoderm of the balstocyst. The functional trophoblast differentiates into villous and extravillous cytotrophoblast, each having a distinct function. The former is multinucleated syncytiotrophoblast which promotes foetal and maternal exchanges and the latter invades the endometrium and spiral arteries. One of the causes of implantation failures is found to be due to the disturbances in the functional components of trophoblast which leads to an early pregnancy loss that is estimated to be nearly 40%. Therefore, the implantation of blastocyst in the uterus depends upon the competence and receptivity of blastocyst and uterine endometrium respectively. The process of implantation includes three phases: (1) apposition, (2) attachment and (3) penetration in a specific time frame. The blastocyst in its free floating sojourn settles on the uterine endometrium and further invasion authenticates the competence of the embryo. Once the invasion is ensured, the endometrial stromal cells undergo decidualization, a response of receptivity from maternal side. Each one of these process involves the role of several cytokines such as LIF, IL-6 and IL-11. The deficiency of these cytokines leads to decrease in viable implantation sites [18]. LIF, a pleiotropic cytokine, induce macrophages proliferation and onsite remodeling of uterine endometrium leading to decidualization [30, 31] whereas LIF mutant mice is defective and fails to initiate implantation. In addition, integrins, matrix metalloprotienases (MMP) and tissue inhibitors of MMP mediate matrix disintegration to the desired extent required for blastocyst implantation and decidualization. All these factors involved in the implantation stage of blastocyst are progesterone dependent. Indian Hedgehog and Wnt signaling pathways are crucial for blastocyst-uterus communication and also for the onset of implantation [5]. Thus, the implantation is a highly coordinated process wherein blastocyst initiates both receptive signals and uterine tissue remodeling.

Foetal Immune system: Foetus is under the protection of maternal immune system. Also, the re-

sponsiveness of foetus towards infection is reasonably low. It does not mean that foetus is free from immune components. The mammalian foetus gradually builds its immune system in a captive sterile uterine environment. To begin with foetus develops tolerance towards the self 'antigens' and maternal antigens. Initially, it modulates to coexist with the maternal immune system and this must have been made plausible due to the incipient T-cells that are 'Triple-negative' ($CD3^-$, $CD4^-$ and $CD8^-$) [14, 15], possibly promotes unresponsiveness towards the maternal antigens. Parallel to this, lymphoid cells start forming from the yolk sac, later shift to foetal liver and ultimately to the bone marrow. Further, lymphoid cells also colonize in the primordial thymus derived from the third visceral arches around six weeks of gestation. Parallel to this, the secondary lymphoid organs provide a temporal space for the naïve immune cells to mature. In addition, mucosal associated lymphoid tissue in gastrointestinal tract and nasal region is at its infancy and soon after parturition mucosal tissues get activated due to the influx of lymphocytes [12]. The most important aspect of suppressive immune response in the foetus would be primarily due to T regulatory lymphocytes [12].

Trophoblast of foetus plays a pivotal role in resisting the maternal aggressiveness towards paternal antigens. Among the cell surface antigens derived from the HLA complex, trophoblast predominantly expresses HLA-G, a non-classical MHC class I, and its isoforms in contrast to maternal tissues. HLA-G is gaining importance because of its role as a specific ligand to multiple receptors of maternal leukocytes and lymphocytes. Evolutionarily, the alleles of HLA-G must have been conserved to support the semi-allograft foetus among viviparous mammals, HLA-G initiates maternal tolerance towards the foetus. Interestingly, unlike HLA- A and B, HLA-G is least polymorphic and restricted mostly to embryonic tissue. In the recent past several reviews [7,9,22] and scientific reports [25,32] elucidated the role of HLA-G in maternal-foetal interface in pregnant viviparous mammals. HLA-G receptors are distributed on maternal immune cells namely NK cells, CD4 and CD8 T cells, B-cell, macrophages and dendritic cells. These receptors are KIR 2DL4, ILT2, ILT4, CD8 and CD160. The interaction of HLA-G ligand with these receptors generates angiogenic factors to build spiral arteries in placenta and immune tolerance against paternal antigens. Therefore, the non-classical HLA-G ligand in mammalian foetus has a major role in promoting to acquire immune privilege and to induce immune tolerance under the canopy of a well sophisticated maternal immune surveillance.

Biomarkers to avert risks in pregnancies: Decidual vascular remodeling is one of the main requirements during implantation of the early embryo. During this time, as indicated in the previous sections, several immunological challenges have to be encountered by both the maternal immune cells and also embryonic trophoblast. The functions of immune cells are always under the influence of a variety of cytokines. The imbalance in cytokines in a pregnant woman leads to risks such as preeclampsia, pre-term birth etc. The cytokines such as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 and IL-10, TNF-α, IFN-γ do interplay and contribute in the safe delivery of foetus (Table 1). The relationship between psychological factors and biomarkers among 49 African American pregnant woman was studied by Carmen Giurgescu *et al*., [33] and reported that the levels of cytokines were found to be altered among hypertensive disorders of pregnancy [34]. The shift in the regulation of immune system towards Th1 cytokine profile was also noticed in preeclampsia, a hypertensive risk during pregnancy due to heterogeneous origin [35]. The evaluation of maternal serum pro-inflammatory cytokine levels and Th1/Th2 response yields prognostic approach to avert the risk during pregnancy. Further, Th1 subpopulations of cytokines, IL-12, IL-18 and IFN-γ have been found to be participated and their evaluation would be the possible diagnostic markers to avert the risk among pre-eclamptic women [36]. In general, maternal serum HLA-G levels may not predict risk in pregnancies; however, contribute to the assessment of a good prognostic indicator for safe pregnancy [9]. Mariana et al., [37] have shown that the systolic and diastolic blood pressure ≥140 and ≥90 mmHg respectively and protein/creatinine ratio ≥ 0.3 are well defined signs in pre-eclampsia.

CONCLUSION

The uterus is a pouch destined for the semi-self foetus to get conditioned and to withstand the maternal immunological challenges. Soon after the process of implantation initiated and mediated by the embryonic trophoblast, the embryo invades the decidua of pregnant uterus successfully and induces maternal immunological tolerance through several mechanisms i.e., expression of HLA-G and 'Triple-negative' T-cells and also promotes on the maternal side the shift towards Th2 cytokine response. How-

Table 1. **Handful of cytokines to evaluate the risk during pregnancy**

Cytokines	Destined roles
IL-1β	Promotes the synthesis of monocyte chemotactic factor in decidual cells
IL-2	Promotes decidual NK and T cells proliferation for building spiral arteries
IL-4	Promotes Th2 response that is predominant in 2nd and 3rd trimesters
IL-5	Promotes Th2 response
IL-6	Potent pro-angiogenic factor
IL-8	Chemoattractant and contributes for spiral arteries formation
IL-10	Protects allo-foetus from rejection during gestation. Also, activates HLA-G expression in embryonic trophoblast cells.
TNF-α	Promotes the synthesis of monocyte chemotactic factor in decidual cells to induce decidual vascularization.
IFN-γ	Trophoblast is insensitive to IFN-γ and hence takes a lead in uninterrupted decidual invasion

ever, the occasional maternal intolerance would be primarily because of the inherent nature of shift towards Th1 cytokine profile possibly culminated to abnormal miscarriages and the same could be maneuvered through the frequent evaluation of maternal serum proinflammatory cytokines.

Acknowledgements

The author acknowledges Department of Science and Technology, Govt. of India, New Delhi for providing financial assistance through a major research project no: SB/SO/ AS -138/2012.

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SERUM PROLACTIN IN MELANOMA PATIENTS WITH INTERFERON ALPHA2B TREATMENT

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ABSTRACT

The authors' interest was focused on prolactin status in patients with melanocytic lesions and on changes induced by interferon treatment in melanoma patients.

Material and method. The study lasted 5 years and included 128 melanoma patients, 48 dysplastic nevi patients and 48 healthy volunteers. Sixty melanoma cases were selected after surgical removal of tumor and divided into 2 groups: 30 patients with 10 $MUmp^{-1}$ interferon alpha2b treatment, three times a week, one year and 30 patients without interferon treatment.

Prolactin assessment was made at inclusion in the study, after surgical removal of tumor, when patients started the treatment, after 1, 6, 12 months of treatment and 6 months after treatment end.

Results. In melanoma patients, high values of prolactin (10.55 ± 5.94 ng/ml) were detected when compared with dysplastic nevi group (5.94 ± 2.87ng/ml) and control group (5.74 ± 3.66ng.ml). Prolactin levels decreased after surgical removal of melanoma, significantly increased during interferon treatment and returned to baseline few months after the immunomodulatory treatment.

Conclusions. The treatment with interferon alpha2b stimulated reversible and non-cumulative prolactin production. Evaluating prolactin in melanoma patients could become necessary in the future, both for finding a possible pituitary disorder, but also for a new pharmacological intervention.

REZUMAT

Interesul autorilor s-a concentrat asupra statusului prolactinei la pacienţii cu leziuni melanocitare şi asupra modificărilor induse de tratamentul cu interferon la pacienţii cu melanom malign.

Material şi metodă. Studiul s-a desfăşurat pe o perioadă de 5 ani şi a inclus 128 pacienţi cu melanoma malign, 48 cu nevi displazici şi 48 voluntari sănătoşi. 60 de cazuri cu melanom malign au fost selectate după excizia chirurgical a tumorii, şi au fost repartizate in două grupuri: 30 pacienţi care au primit tratament cu interferon alfa2b 10MU/mp, de 3 ori pe săptămână, timp de un an şi 30 pacienţi fără tratament.

Determinarea prolactinei a fost realizată la includerea în studiu, după excizia chirurgicală a tumorii, la începutul tratamentului, după 1,6,12 luni de tratament şi la 6 luni de la încheierea tratamentului.

Rezultate. La pacienţii cu melanom malign, valori crescute ale prolactinei (10.55±5.94 ng/ml) au fost detectate, în comparaţie cu grupul de pacienţi cu nevi displazici (5.94±2.87ng/ml) sau grupul control (5.74±3.66ng.ml). Nivelurile serice ale prolactinei au scăzut după excizia chirurgicală a melanomului şi au revenit la nivelul de bază la câteva luni după tratamentul imunomodulator.

Concluzii. Tratamentul cu interferon alfa2b a stimulat reversibil şi non-cumulativ producţia de prolactină. Evaluarea prolactinei la pacienţii cu melanom ar putea deveni necesară în viitor, atât pentru a determina o posibilă afectare pituitară, cât şi pentru o intervenţie farmacologică.

Keywords: prolactinemia, malignant melanoma, immunomodulatory treatment

INTRODUCTION

Several experimental and clinical studies sustain that immune-endocrine system might be involved in tumorigenesis. Malignant melanoma is a challenging disease for researchers due to unknown hormonal disorders involved in this type of cancer. Little information about endocrine changes produced by interferon alpha treatment in melanoma is available.

Taking into account that melanoma is a hormone-sensible cancer [1], authors aimed to analyze hormonal status in patients with melanocytic lesions and changes induced by interferon treatment in melanoma.

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Nowadays, the studies showed that biological activity of prolactin is not limited at reproductive system, it also has important functions in other tissues and systems not known as hormone-sensible [1, 2, 3, 4]. Identifying prolactin in skin (keratinocytes, melanocytes, hair follicle, dermic fibroblasts, macrophages, endothelial cells, adipocytes, lymphocytes, skin tumors) was a major argument for studying prolactin involvement in cutaneous disorders. Moreover, secretion of prolactin in the skin is regulated by some neuroendocrine mediators [3, 5, 6].

Control mechanisms of cutaneous secretion and expression of prolactin are still unknown. Regulation of pituitary prolactin transcription and secretion is different from that of extrapituitary prolactin [3, 4, 5, 6, 7, 8, 9, 10]. Pituitary specific transcription factor PIT-1 is essential for pituitary secretion of prolactin. Extrapituitary secretion of prolactin seems to be PIT1-independent [7].

Secretion and expression of pituitary prolactin are influenced by physiological factors (pregnancy, breastfeeding, nipples stimulation, sexual act, physical effort, physical or psychical stress), by pharmacological factors (ocitocin, histamine, serotonin, estrogens, VIP - vasoactive intestinal peptide, dopamine, IMAO - monoamine oxidase inhibitor, endoteline-1, TGF-1 - transforming growth factor1, calcitonin, bFGF - basic fibroblast growth factor, EFG-1 - epidermal growth factor1, vasopresin, angiotensin-2, agonists and antagonists of dopamine, opioids, anesthetics, antihistamine, NPY, galanine, P substance, neurotensin, bombesin-like peptides, acetylcholine, interleukin 6), pathological factors (pituitary disorders, irradiation, medullar lesions, thoracic lesions, severe hepatic disorders, renal injury). Extrapituitary prolactin is not stimulated by TRH or estradiol and is not inhibited by dopamine or bromocriptine [8]. Progesterone does not induce prolactin synthesis in human cutaneous fibroblasts *in vitro*, while prostaglandin 2 stimulates prolactin secretion [9].

Prolactin can be found in circulation under different forms after *immunological* characteristics and *biological* activity. Though, we can find in humans the intact molecule of 23kDa and, also, other isoforms of prolactin (14kDa, 16kDa, 22kDa, 48kDa, 150kDa). Prolactin of 23 kDa has angiogenic properties, while prolactin of 16kDa seems to be antiangiogenic, antilymphangiogenic and antiapoptotic [4, 10, 11]. In tissues, prolactin acts through receptors that activate the signaling pathway JAK2/STAT5a/b, MAPK and PI3K [12]. Receptors of prolactin were found in keratinocytes, fibroblasts, sebaceous glands, skin immune cells [2, 3, 4, 5, 6, 13].

Prolactin synthesized in lactophor acidophil cells from pituitary gland acts as hormone on organs situated at distance from the site of its secretion, while extrapituitary prolactin acts locally as cytokine [10]. Importance of prolactin in initiation and progression of cancer was underestimated for long time. Recent researches showed that prolactin was involved in tumor genesis, immune anti-tumor response of macrophages, tumor cells proliferation, differentiation, motility and migration, apoptosis, angiogenesis, lymphangiogenesis, survival, resistance to treatment [4, 11, 12, 13, 14, 15]. Recent researches showed extrapituitary prolactin role in mammary cancer [16, 17, 18, 19], prostate cancer [20, 21], colorectal cancer [22], gynecologic cancer [23], laryngeal cancer [24], hepatocellular carcinoma [14], gastrointestinal cancer [21], hematopoietic cancer [21], melanoma [25].

Prolactin was considered risk factor in mammary cancer [16], by influence on survival, proliferation, apoptosis, motility of mammary tumor cells, and, resistance to chemotherapy (cisplatin, paclitaxel) [17]. Prolactin determined apoptosis, increased cytotoxic effects of doxorubicin and paclitaxel *in vitro* and inhibited tumor genesis [18]. Overexpression of prolactin and its receptors in prostate cancer and *in situ* carcinoma, high correlations between serum prolactin and disease severity, STAT5 activity and Gleason score sustained prolactin as a major risk factor in prostate cancer and in resistance to treatment [20, 21]. High prolactinemia by JAK2 pathway could contribute to hepatocarcinoma development [14], could promote gynecological cancers by activating oncogene Ras and altering suppressing genes of tumor genesis [23].

Prolactin role in melanogenesis was intensively studied. In a previous study, the authors evaluated prolactin in patients with malignant melanoma before and 8 weeks after surgical removal of the tumor. High levels of prolactin were detected in patients with melanoma, levels that decreased after surgical removal of tumor. These results sustain the idea that prolactin is an active participant in melanoma development [26].

The authors aimed to evaluate prolactin status in patients with melanocytic tumors and its serum changes produced by interferon alpha2b (IFNa2b) treatment in melanoma. Many trials evaluated the effect of IFNa2b in progression of melanoma, in the last years melanoma being the most used as adjuvant

therapy, after surgical removal of tumor in this type of cancer [27].

MATERIAL AND METHODS

The study lasted five years and was based on a prospective analysis of 128 adults with early stages of melanoma (stage 1 and 2 according American Joint Committee of Cancer), 48 adults with dysplastic nevi and 48 healthy volunteers. The melanocytic lesions were first identified, surgically removed and histopathologically analyzed. The study did not include patients under 18 years old, women under 50 years with premenopausal manifestations, patients with infectious disease, pregnant women, drug or alcohol abuse, psychiatric disorders, ischemia, hepatic diseases, chronic renal diseases, prolactinoma, metabolic disorders or other endocrine disorders.

All the subjects in the study were diagnosed using clinical, paraclinical, imagistic criteria. All the patients signed the free informed consent according to the declaration of Helsinki 1964. In women, we determined prolactin for premenopausal ones (aged < 50 years) and postmenopausal ones (aged > 50 years).

Prolactin was determined by chemiluminescence using Access Immunoassay Systems. Prolactin present in the samples binds to the anti-prolactin coated microparticles. After washing, anti-prolactin acridinium labeled conjugate is added in the second step. Pre-Trigger and Trigger Solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs).

The venous blood was collected in the morning before eating in vacutainers. After 30 minutes, the blood was centrifugated at 3500 rotations/min for 10 minutes.

The samples were obtained as follows:

- For control group - a single evaluation when included in the study (0 moment);
- For dysplastic nevi group - two evaluations when included in the study (0 moment) and 8 weeks after surgical removal (1st moment);
- For melanoma – a group evaluation when included in the study (0 moment), 8 weeks after surgical removal (1st moment). 60 individuals were selected from high risk melanoma patients (30 patients received immunotherapy with IFNa2b, $10MUxmp^{-1}$, 8 weeks after surgical removal, third times per week for one year, and 30 patients that did not receive IFNa2b). The patients were evaluated before the surgical removal (0 moment), before beginning of immunomodulatory treatment (1st moment), at 1 month (2nd moment), 6 months (3rd moment) and 12 months (4th moment) during the treatment and 6 months after immunomodulatory treatment (5th moment).

All the results were analyzed using SPSS, a software for statistic evaluation. The results were presented as mean±standard deviation. The variation between groups was evaluated using ANOVA test. $p<0,05$ was considered with statistical significance.

RESULTS

We evaluated prolactin status in melanoma patients compared with dysplastic nevi patients and with control. The groups were similar for age, sex and biological profile as shown in Table 1. Prolactin levels increased statistically significant in melanoma patients compared with dysplastic nevi group (10.55 ± 5.94 ng/ml versus 5.94 ± 2.87 ng/ml, $p < 0.001$, CI 95%) and control group (10.55±5.94 ng/ml versus 5.74 ± 3.66 ng/ml, $p < 0.001$, CI 95%). The same statistical variations were observed when analyzing prolactin variation by sex and age. In premenopausal women from melanoma group, prolactin level was 14.11 ± 6.41ng/mL, significantly increased when compared with control group (9.06 ± 4.16 ng/ml). In melanoma postmenopausal women, prolactin level was 10.07 ± 4.23 ng/ml, significantly increased when compared with control group (5.16 ± 3.11 ng/ml). Prolactin increased statistically significant in men with melanoma (6.33 ± 3.15 ng/ml) versus control (3.02 ± 2.26 ng/ml).

Prolactin did not vary statistically significant with histopathological features (site tumor, histopathological type, Clark level, Breslow index) in melanoma group.

Prolactin levels decreased after surgical removal of the tumor. Prolactin was significantly reduced by 11.4% compared with the moment of surgical removal in melanoma patients (9.23 ± 5.43 ng/mL versus 10.55 ± 8.5 ng/mL, IC = 95%, $p < 0.001$) and did not vary significantly in dysplastic nevi group (5.94 ± 2.87 ng/mL versus 5.90 ± 2.20 ng/ml CI 95%, $p>0.05$) 8 weeks after surgical removal of tumor as presented in Fig. 1.

Prolactin status in melanoma patients during IFNa2b treatment and in melanoma patients without it was presented in Table 2.

In premenopausal women treated with IFNa2b, prolactin had a statistically significant increase ($p<0.001$, IC = 95%) by 44.65% after 1 month, 28.22% after 6 months, 34.31% after 12 months of immunomodulatory treatment comparing with the

Table 1. **Demographic and biological characteristics of patients with melanocytic tumors and control at the inclusion in the study (before surgical treatment of melanoma)**

Characteristics	Melanoma (n = 128)	Dysplastic nevi (n = 48)	Control (n = 48)
Sex: men/women	1:1.46	1:1.40	1:1.28
Age (years)	52.92±13.48	50.52±14.03	51.75±13.43
BMI (kg/m^2)	23.8±1.9	24.1±1.6	23.3±1.4
Hemoglobin (g/dl)	12.6±1.3	12.9±2.1	13.1±1.8
Leukocytes (cel/mmc)	5400±1100	5650±935	5710±982
Platelets x10^3(/mmc)	237±31	286±93	254±87
Glucose (mg/dl)	88.3±16.2	91.5±12.4	83.7±9.6
ALT (U/L)	19.4±6.2	21.8±8.3	18.1±7.9
AST (U/L)	20.4±8,3	19.2±5.7	19.9±4.3
Cholesterol (mg/dl)	150±30.8	178±24.5	164±33.9
HDL-cholesterol (mg/dl)	47.4±8.1	51.9±9.5	49.7±7.9
Triglycerides (mg/dl)	81.4±22.9	77.2±19.4	72.1±11.4
Urea (mg/dl)	26.1±9.3	31.7±12.3	28.9±10.4
Creatinine (mg/dl)	0.81±0.14	0.78±0.09	0.75±0.12
Prolactin (ng/ml)	10.55±8.50	5.94±2.87	5.74±3.66

n= number of patients

initial moment (before beginning IFNa2b). Six months after IFNa2b treatment, prolactin had the same values as at the initial moment. In postmenopausal women, prolactin dynamic under IFNa2b was similar; statistically significant increase by 29.25% after 1 month ($p<0,01$, CI 95%), by 9.66% after 12 months of treatment ($p<0.05$, CI 95%), compared with the initial values of prolactin. Prolactin variations in subgroups without IFNa2b treatment were insignificant. In men with melanoma, statistically significant increases of prolactin ($p<0.01$, CI 95%) were observed after 1 month (by 37.05%), after 6 months (by 32.90%) and also after 12 months (by 9.52%) of treatment compared with the initial moment. Prolactin did not vary in melanoma men without treatment.

The dynamics of serum prolactin in all patients treated with IFNa2b (30 cases) presented in Fig. 2, confirmed the analysis shown before. IFNa2b stimulated the synthesis of prolactin in melanoma patients, a reversible situation 6 months after the treatment end. During the study, no patients died and no tumor recurrence was observed.

DISCUSSION

The data of the study sustained the hypothesis that malignant tumors produced large amounts of

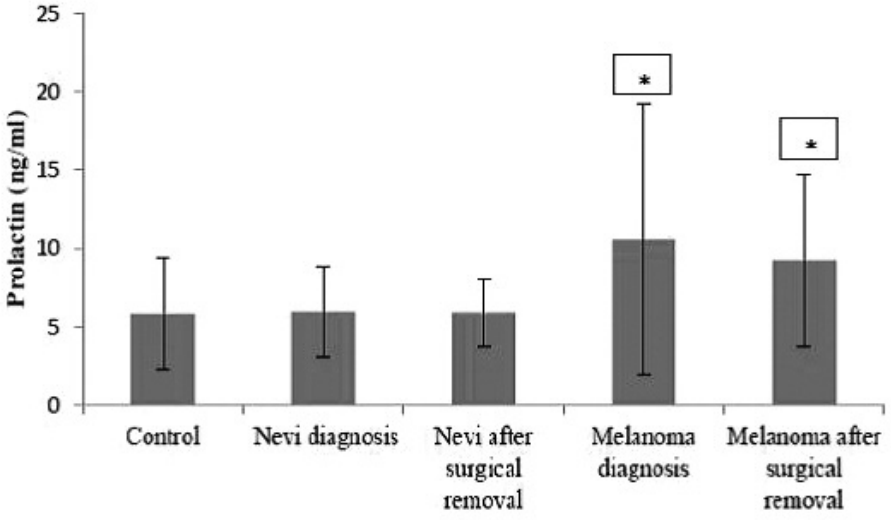

Fig. 1. Prolactin variation in patients with melanocytic tumors before and after surgical removal (*statistical significance)

Table 2. **Variations of prolactin serum levels in patients with malignant melanoma during the treatment with IFN alpha 2b**

Subgroups	Prolactin (ng/ml) in women under 50 years	Prolactin (ng/ml) in women over 50 years	Prolactin (ng/ml) in men
Without IFN (n = 30)			
1	8.30±4.59	11.32±6.38	7.40±2.50
2	8.61±4.64	11.07±6.15	7.39±2.39
3	8.17±3.18	11.09±9.29	7.56±2.78
4	8.18±3.92	11.52±6.25	7.21±2.93
5	8.55±4.49	10.76±5.80	7.22±2.92
Treatment with IFN (n = 30)			
1	10.95±4.26	10.12±5.89	8.61±3.80
2	15.84±4.00	13.08±8.45	11.80±5.00
3	14.04±4.15	10.47±9.17	11.84±5.08
4	14.7±4.45	10.98±7.54	9.43±4.06
5	9.71±2.83	10.11+6.89	8.71±3.68

n= number of patients

prolactin. First of all, prolactin was statistically significant increased in melanoma patients compared with dysplastic nevi patients (melanoma prolactin: dysplastic nevi prolactin ratio was 1.88), with control group (melanoma prolactin: control prolactin ratio was 1.93). Other arguments were: low levels of prolactin 8 weeks after surgical removal of melanoma compared with its levels before operation, insignificant variation of prolactin in dysplastic nevi.

Recent researches showed extrapituitary prolactin role in mammary cancer [16, 17, 18, 19], prostate cancer [20, 21], colorectal cancer [22], gynecologic cancer [23], laryngeal cancer [24], hepatocellular carcinoma [14], gastrointestinal cancer [21], hematopoietic cancer [21], melanoma [25].

Prolactin was involved in modulating melanin system [25], in hypermelanosis ethiopathogenity [28], in melanoma and mammary cancer angiogenesis [29, 30]. Prolactin was accumulated in melanoma cells and influenced cell cycle, proliferation and response of melanocytes exposed to UV. Prolactin induced transcription of cycline D, which interacted with CDK4 and CDK6 in G1 phase of cell cycle. Prolactin stimulated cell cycle progression in melanoma, mitotic activity, high levels of cytosolic calcium, regulated transition G1/S and S and M phases, tumor angiogenesis and cell differentiation [29]. Human prolactin of 16KDa was considered an inhibitor of angiogenesis both *in vivo* and *in vitro* [11]. This effect was demonstrated by gene transfer

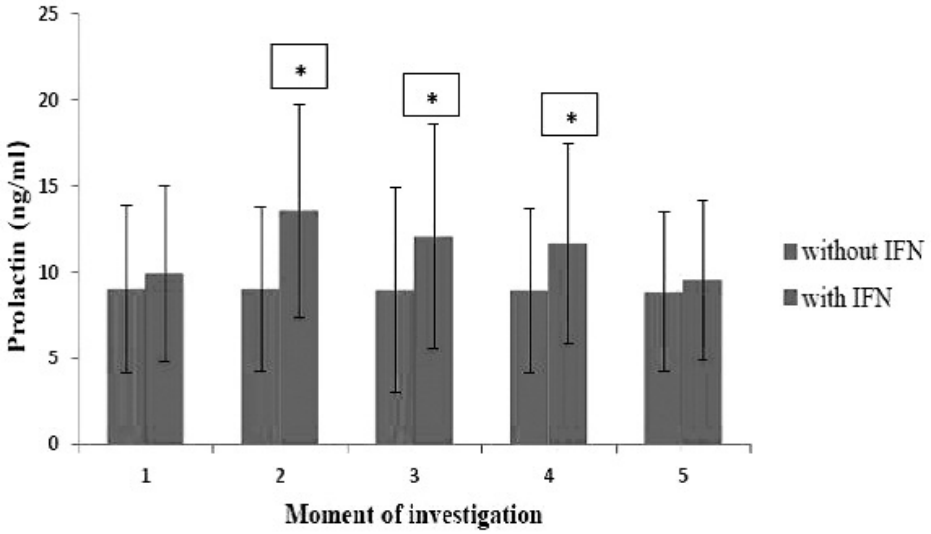

Fig. 2. Prolactin dynamic in melanoma patients treated with IFN versus melanoma patients without treatment (*statistical significance)

method based on cationic liposomes to produce 16Kh prolactin in B16F1O mice. This experiment showed that neovascularization and tumor development were significantly reduced in melanoma [11].

Based on the obtained correlations between prolactinemia and melanoma histopathological characteristics, it is hard to appreciate if prolactin dysfunction was an early process or a consequence of tumor process. Some arguments for prolactin implication in histopathological evolution of melanoma are stustained by studies that showed prolactin stimulated releasing of specific cytokines (IL2, IFNγ) by Th1 cells, inhibited apoptosis [31, 32] and amplified angiogenesis [33, 34, 35].

Another interesting fact in this study was the increase of prolactinemia in melanoma patients treated with IFNa2b (Table 2). Taking into account the reversibility of this phenomenon, the authors associated prolactin variation with immunomodulatory treatment. Recent studies considered prolactin to have an essential role in immune response control during therapy with IFNa2b [36, 37]. It seemed that prolactin activated signaling pathway JAK/STAT and stimulated IRF1 (interferon regulating factor 1). Moreover, prolactin influenced Th1, Th2, other T helper cells, T regulating cells. Pituitary dysfunctions were rarely during the treatment with IFNa2b and they might be associated with genetic susceptibility [36].

Prolactin influenced antitumor immune reactions of macrophage and induced IL-12 releasing [33], phenomena that could be inhibited by interleukin 4. Prolactin increased hemoxygenases-1 expression and induced VEGF production in human macrophages [34]. Melanoma cells secreted proinflammatory cytokines that inhibited cells response to IFN alpha/beta by altering its receptors [35]. Thus, complex inflammatory processes that included prolactin, correlated with vascular endothelial dysfunction could lead to a proinflammatory and proangiogenic microenvironment that could influence melanoma progression.

CONCLUSIONS

Low levels of prolactin after surgical removal of the tumor sustained the possible implication of prolactin in melanoma development. The treatment with IFNa2b stimulated prolactin production, but this was a reversible and non-cumulative effect.

Reducing tumor growth by suppressing prolactin production or by blocking prolactin receptors could be an idea for developing new antitumor agents.

Acknowledgements

There are no conflicts of interest between the authors of the paper.

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SEROLOGICAL RESPONSE TO VACCINES IN CHILDREN WITH DIABETES

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ABSTRACT

Objective: Patients with diabetes mellitus (DM) are more susceptible to infections. Deficiency in some domains of immune system could be one of the main reasons, which increases the risk of infections. The aim of this study was to assess antibody responses to vaccines in a group of children with diabetes and in the controls.

Methods: a cross-sectional study was performed among 90 children under 15 years of age with a history of type 1 DM, referred to endocrinology clinics of university hospitals; Mofid Children Hospital and Loghman Hospital. Also, we enrolled ninety healthy children as the control group. Antibody levels against diphtheria, tetanus, pertussis, measles, mumps, rubella and hepatitis B (HB) were measured by enzyme-linked immunosorbent assay (ELISA).

Results: Among 90 patients with diabetes, 48% were male and 52% were female and in the control group 49% were male and 51% were female. Regarding IgG antibody levels against measles, there was not any significant difference between the two groups, but according to the applied kit, IgG levels against measles vaccine were positive in 62% of the diabetic and 84% of the controls. Also, there was a significant difference between the two groups in terms of IgG antibody level against rubella, but consistent with the applied kit, there was not any significant difference between the two the groups.

Conclusion: Given the results of the study, no difference was found between patients with diabetes and controls who were vaccinated with pertussis, diphtheria, tetanus, mumps and HB vaccines. But there are some concerns about measles and rubella vaccinations that need further investigation.

REZUMAT

Obiectiv: Pacienţii cu diabet zaharat (DZ) sunt mai susceptibili la infecţii. Unul dintre motivele principale ar putea fi constituit de anumite deficienţe ale sistemului imunitar care cresc riscul de infecţie. Scopul acestui studiu este evaluarea răspunsului în anticorpi la vaccinuri la un grup de copii cu diabet şi la grupul martor.

Metode: s-a efectuat un studiu transversal pe un număr de 90 de copii cu vârsta mai mică de 15 ani şi istoric de DZ tip 1, internaţi în clinici de endocrinologie din spitalele universitare Mofid şi Loghman. De asemenea, au fost incluşi în studiu 90 de copii sănătoşi, ca grup martor. Nivelele de anticorpi anti difterie, tetanos, pertussis, rujeolă, oreion, rubeolă şi hepatită B (HB) au fost măsurate prin metoda imunoenzimatică (ELISA).

Rezultate: Din cei 90 de pacienţi cu diabet, 48% erau de sex masculin şi 52% de sex feminin, în timp ce în grupul martor 49% erau de sex masculin şi 51% de sex feminin. În ceea ce priveşte nivelele de anticorpi IgG anti rujeolă, nu a existat nici o diferenţă semnificativă între cele două grupuri, dar, conform kit-ului utilizat, nivelul anticorpilor IgG anti rujeolă a fost pozitiv la 62% din grupul de pacienţi cu diabet şi 84% din grupul martor. De asemenea, a existat o diferenţă semnificativă între cele două grupuri în ceea ce priveşte nivelul anticorpilor IgG anti rubeolă, dar, conform kit-ului folosit, nu a existat o diferenţă semnificativă între cele două grupuri.

Concluzie: Având în vedere rezultatele studiului, nu s-a observat nici o diferenţă între pacienţii cu diabet şi membrii grupului martor vaccinaţi pentru pertussis, difterie, tetanos, oreion şi HB. Există însă o anumită îngrijorare privind vaccinarea pentru rujeolă şi rubeolă care necesită investigaţii suplimentare.

Keywords: vaccine, pertussis, diphtheria, tetanus, measles, rubella, mumps, diabetes mellitus

Abbreviations: DM - Diabetes mellitus, PMN - polymorphonuclear, HB - hepatitis B

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1. INTRODUCTION

Diabetes mellitus (DM), as one of the major threats to health in the 21st century [1], is among the most common endocrine diseases of children. According to estimations, there will be 380 million patients with DM in 2025 [2]. As reported by Diabetes Atlas, there are about 490,100 under 15 year children living with type 1 diabetes and it was also estimated that in 2011 about 77,800 individuals under 15years developed the disease [3]. Some published reports emphasize the increasing trend of diabetes in children [4-7].

Frequent rate of infections in DM has been often reported [8-11], as supported through a research that disclosed the increased rate of lower respiratory tract infection, urinary tract infection, and skin and mucous membrane infection in patients with diabetes [10]. About half of the patients had at least one episode of infectious disease leading to hospitalization or referral to a physician in each studied year [11]. Some rare infections like mucormycosis, malignant external otitis, emphysematous pyelonephritis and emphysematous infections of the gall bladder, kidney, and urinary bladder are seen, especially in the presence of diabetes [11, 12]. One factor that may explain the increased rate of infections in the diabetes is an altered immune system function [13, 14].

There are some reports that show some varied immune processes in diabetes [13, 15, 16]. A major concern is regarding the decreased or compromised function of polymorphonuclear (PMN) and monocytes/ macrophage cells. Phagocytosis, leukocyte adherence and chemotaxis may be altered in DM [14]. Decreased PMN leukocyte function is predominant in the presence of acidosis [15, 17, 18].

Other domains of the immune system like antioxidant systems and humoral immunity also may be depressed functions in people with diabetes [15, 19]. Additionally, impairment of cutaneous responses to antigen tests and T-lymphocyte function and blastogenesis [14, 19-21], disorders of humoral immunity and poor antibody response [10, 13, 20, 21], deficiency of the C4 component [13, 22, 23] that could be associated with PMN dysfunction and decreased cytokine production, decreased secretion of interleukin-1 (IL-1) and IL-6 after stimulation tests [13, 20], decreased CD4/CD8 lymphocyte ratios and alterations in natural killer cell function, were also reported in patients with diabetes[21]. It seems that increased glucose level in the blood can impair the humoral immunity and lymphocyte functions; a phenomenon that can be inverted by normalization of blood glucose levels [15]. Furthermore http://reference.medscape.com/medline/abstract/9017350, increased glycation may prevent the production of IL-10 by myeloid cells, impair interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α production by T cells and deteriorate cell immunity by reduced expression of class I major histocompatibility complex (MHC) on the surface of myeloid cells. Reduced T cells response [20, 24] and lower production of interleukins [24, 25] could be observed in diabetic patients.

The aforementioned conditions raise concern regarding the immune response to vaccines in the presence of diabetes. Although there is reported research that shows adequate antibody response to influenza, pneumococcal, and hepatitis B (HB) vaccinations [26-28], there are no enough reports on the response to other vaccines like pertussis, diphtheria, tetanus, measles, rubella and mumps, particularly in children with diabetes. Therefore, the aim of this study was to assess the antibody levels of the vaccines in children with diabetes and compare them with the controls.

2. PATIENTS AND METHODS

2.1. Patients

This cross-sectional, descriptive and analytical study was conducted in the endocrinology clinics of university referral hospitals including Mofid Children Hospital and Loghman Hospital. Ninety children with a history of DM type 1, referred to the clinics for routine checkup, were selected sequentially to enroll in the study. Ninety healthy children were also included in the survey as a control group. Among 90 patients with diabetes, 48% were male and 52% were female and in the control group, 49% were male and 51% were female. The mean ages of the patients and control group were 8.6 ± 2.6 (2.5-9) and 8.5 ± 2.6 (3-9), respectively. The minimum ages in the diabetic and control group were 2.5 and 3 years, respectively (Table 1). The maximum age in both groups was 9 years.

DM was defined with the following criteria:

1. Fasting blood sugar of 126 mg/dl or more.
2. Random blood sugar of 200 mg/dl or more plus symptoms like polydipsia or polyuria.

Inclusion criteria:

1. All children under 15 years referred to the endocrinology departments of Loghman and Mofid Children hospitals.
2. Documented DM.

Table 1. **Mean of age and sexual distribution of control and diabetic patients**

Gender	Group	Mean of age	Percent of sample (%)
Male	Case	8.6± 2.7	48
	control	8.5± 2.7	49
Female	Case	8.6± 2.6	52
	Control	8.5± 2.6	51

3. Absence of other chronic diseases or known immunodeficiency disorders.
4. Receiving routine national immunization.

Exclusion criteria:

1. The use of blood products within the past 3-6 months.
2. Hospitalization within 3 months.
3. Chronic diseases other than diabetes type 1.
4. Proven Immunodeficiency.
5. Unknown vaccination status.
6. Severe malnutrition.

The routine vaccination schedules in our country at the research time consist of the following: (1) at birth: BCG, Hepatitis B, Polio, (2) 2 months of age: Hepatitis B, Polio, DTP, (3) 4 months of age: Hepatitis B, Polio, DTP, (4) 6 months of age: Hepatitis B, Polio, DTP, (5) 12 months of age: MMR, (6) 18 months of age: MMR, Polio, DTP, (7) 4-6 years of age: Polio, DTP.

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study. All patients' guardians had signed informed consent forms before the sampling. Totally, 90 healthy children were enrolled in the study as a control group with no chronic illnesses.

2.2. Laboratory methods

Patients' epidemiologic and demographic data were registered in a short questionnaire. Three milliliters of venous blood was collected from each patient and control and plasma was isolated immediately and stored at −80 °C until use at the reference laboratory in the Pediatrics Infection Research Center of Mofid Children Hospital. Antibody levels against diphtheria, tetanus, pertussis, measles, mumps, rubella and HB were measured in samples by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (eBioscience, Austria, Europe).

2.3. Statistical analysis

The quantitative variables were compared using student's t-test, while the categorical ones were compared through Chi-square and Mann-Withney test. Quantitative variables were expressed as mean ± SD. A P-value < 0.05 was deemed statistically significant and analysis was performed using SPSS version 16. Student's t-test and Chi-square test were used to compare the antibody levels in the two groups.

3. RESULTS

In this study, we evaluated the antibody response to diphtheria, tetanus, pertussis, measles, mumps, rubella and HB vaccines in 90 diabetic patients and controls. Basic demographic data revealed no significant difference between the two groups in mean age and gender. The mean duration of diabetes was 2.1 ± 1.2 years.

The antibody levels against pertussis and tetanus vaccines were assessed in 48 patients with diabetes and 43 controls. Level of antibody against diphtheria vaccine was examined in 43 patients with diabetes and 35 controls.

The mean level of IgG antibody against diphtheria vaccine was 0.9 ± 0.28 IU/ml in patients and 0.86 ± 0.32 IU/ml in the controls. Statistical analysis showed no significant difference between the two groups (P value= 0.74). The mean levels of IgG antibody against tetanus in the diabetic and control groups were 2.8 ± 2.1 IU/ml and 3.1 ± 2 IU/ml, respectively, with no statistically significant difference (P value = 0.94). Levels of antibodies to pertussis vaccine were in the positive range in 50% and 58% of the patients and controls, respectively, according to the applied kit cutoff points (positive, negative), without any significant difference between the two groups (P value = 0.44) (Table 2).

IgG antibody levels against rubella were measured in 89 and 90 of patients with diabetes and control group, respectively. Average IgG antibody levels against rubella vaccine in patients and controls were 81±26.5 IU/ml and 73.6±24.1 IU/ml, respectively, with significant statistical difference between groups (P value<0.001).

According to the applied kit cutoff points (positive, negative), IgG antibody level against rubella vaccine was positive in 88% of the patients and 90%

***Table 2*. Characteristics of antibody response to diphtheria, tetanus, pertussis, measles, mumps, rubella and hepatitis B vaccination in children with diabetes and controls**

vaccine	Diabetics	Control	P value
diphtheria Case = 43 Control = 35	Mean of IgG level (IU/ml) = 0.9 ± 0.28	Mean of IgG level (IU/ml) = 0.86 ± 0.32	0.74
	Protective level defined by WHO = 100%	Protective level defined by WHO = 100%	1.00
Tetanus Case = 48 Control = 43	Mean of IgG level (IU/ml) = 2.8 ± 2.1	Mean of IgG level (IU/ml) = 3.1 ± 2	0.94
	Protective level defined by WHO = 100%	Protective level defined by WHO = 100%	1.00
Pertussis Case = 48 Control = 43	Positive by kit = 50%	Positive by kit = 58%	0.44
Rubella Case = 89 Control = 90	Positive by kit = 88%	Positive by kit = 90%	0.31
	Mean of IgG level (IU/ml) = 81±26.5	Mean of IgG level (IU/ml) = 73.6±24.1	0.001
Measles Case = 89 Control = 90	Positive by kit = 62%	Positive by kit = 84%	0.001
	Mean of IgG level (IU/ml) = 60 ± 52	Mean of IgG level (IU/ml) = 64.8 ± 43.1	0.38
Mumps Case = 90 control = 90	Mean of IgG level (IU/ml) = 115.8 ± 70.5	Mean of IgG level (IU/ml) = 135.6 ± 83.5	0.07
	Positive by kit = 81%	Positive by kit = 81%	1.00
Hepatitis B Case = 90 Control = 90	Protective level defined by WHO = 54%	Protective level defined by WHO = 47%	0.29
	Mean of IgG level (IU/ml) = 45/9 ± 66	Mean of IgG level (IU/ml) = 63/7 ± 83/2	0.113

of control group, with no significant difference between them (P value= 0.31) (Table 2).

IgG antibody level against measles was measured in 89 and 90 of the diabetic patients and controls, respectively. Average IgG antibody level against measles in the former was 60±52 IU/ml and in the latter was 64.8±43.1 IU/ml, without any significant difference between the two groups (P value=0.38).

According to the applied kit cutoff points (positive, negative), IgG antibody level against measles vaccine was positive in 62% of the diabetic and 84%of control group, with a significant difference between them (P value<0.001) (Table 2).

IgG antibody level against mumps was measured in all (90) cases of diabetic and control groups. Average IgG antibody levels against mumps vaccine in the patients and controls were 115.8±70.5 IU/ml and 135.6±83.5 IU/ml, respectively. There was no significant difference between the two groups (P value = 0.07).

According to the applied kit cut off points (positive, negative), IgG antibody levels against mumps vaccine in 81% of the diabetics and 81% of controls were positive, with no significant difference between them (P value=1.00) (Table 2).

IgG antibody level against HB was measured in all (90) individuals of diabetic and control groups. The average antibody levels of HB antibody in diabetic group and in control group were 45/9±66 IU/ml and 63/7±83/2 IU/ml, respectively, without any significant difference between the two groups (P value=0.113).

The members of each group were divided into two subgroups of those with antibody in protective

range and those with insufficient immunity against HB. The levels higher than 10 for this antibody were considered as protective range. Accordingly, 54% of diabetic group and 47% of control group had HB-antibody level within protective range, without any significant difference between the two groups (P value= 0.29) (Table 2).

4. DISCUSSION

Increased occurrence of infections in patients with diabetes has been a matter of concern in practice and there are several published data that display or at least under question the immune system function in diabetics. One of the most critical issues regarding the integrity of the immune system is the serological response to vaccines. There have been no published data about assessment of the antibodies after diphtheria, tetanus and pertussis vaccination in the diabetics. According to WHO position papers, after diphtheria vaccination, antitoxin levels below 0.01 IU/ml are non-protective, levels of 0.01 IU/ml may provide some protection and concentrations of 0.1 IU/ml or more are considered fully protective [29]. In the present study, all of the diabetic and control groups had diphtheria antitoxin concentrations of 0.1 IU/ml or more. Besides, mean of anti-diphtheria antibodies was not different between the diabetics and controls, indicating the satisfactory seroimmunity of the diphtheria vaccine in these patients (Table 2).

According to WHO position papers, if standard ELISA methods are used to measure the anti-tetanus antibodies, the antitoxin concentrations of at least 0.1– 0.2 IU/ml are designated as positive [30]. According to our results, all the patients and controls had anti-tetanus antibody titer of 0.1 or more. The mean of the anti-tetanus antibodies also, was not lower in children with diabetes. This denotes the adequate seroimmunity of the tetanus vaccine in children with diabetes (Table 2).

The currently used whole-cell (wP) vaccines in the world are different and with heterogeneous components. Furthermore, there are no accepted or well defined serological criteria that indicate protection. The wP vaccines induce a higher Th1 and Th17 responses, compared to aP vaccines that induce more Th2 responses. The percentages of the children with positive anti-pertussis antibody level were not significantly different in diabetics, compared with the control group, which suggests the acceptable function of the pertussis vaccine in children with diabetes.

Protection against measles, mumps and rubella in Iran is via administration of MMR vaccine, which is a live attenuated combined vaccine. Measles vaccine causes the activation of cell-mediated immune responses through the production of virus-specific $CD4^+$ and $CD8^+$ T lymphocytes and eventually induces humoral and cellular immune responses, in a similar way after a natural infection. Measles immunization produces both serum and mucosal antibodies.

We found only one published study regarding the assessment of MMR vaccine as a whole, in the patients with diabetes. In Hiltunen' study a group of children with diabetes was compared with two other groups; a group of their siblings and other non-related age- and sex-matched individuals without diabetes, in terms of the MMR directed antibodies. All groups had received MMR vaccine. Only mumps antibodies were significantly lower in the group with diabetes. They concluded that because of a similar risk of exposure in all groups, the lower antibody level in patients with diabetes could be explained by decreased responsiveness to the mumps vaccine [31]. We did not find any difference between the diabetics and non-diabetics, regarding the level of anti-mumps antibody.

HB vaccine induces protection after three primary doses in more than 95% of recipients. Its protection is the result of producing effective anti-HBs antibodies and induction of host's memory T-cells as well.

An anti-HBs concentration of 10 mIU/ml is considered protective to infection with HB virus [32, 33]. There was no difference in the anti-HBs levels among children with diabetes and the controls in our study. Likewise, the immune response of HB vaccine in persons with diabetes has been evaluated recently in the form of a systematic review of the literature [34]. According to the review conclusion, children and young adults with diabetes have similar immune responses after standard administration protocols of HB vaccine, when compared with age-matched individuals without diabetes.

Acknowledgements: We would like to acknowledge the Pediatric Infections Research Center affiliated to Shahid Beheshti University of Medical Sciences, Tehran, Iran for their support of this work. Our thanks also go to Hassan Khajehei, for copy editing of the manuscript.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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