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African Journal of Pharmacy and Pharmacology

Review

Advances in the study of the biological function of Daxx

Jinhong Liu[1]*, Guozuo Xiong[2], Duanfang Liao[1, 3], and Qinhui Tuo[1]

[1]Learning Key Laboratory for Pharmacoproteomics, School of Life Science and Technology, University of South China, Hengyang 421001, China.
[2]Department of General Surgery, The Second Affiliated Hospital in University of South China, Hengyang 421001, China.
[3]Department of Traditional Chinese Diagnotics, School of Pharmacy, Hunan University of Chinese Medicine, Changsha, 410208.

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Death domain-associated protein (Daxx) was originally identified as a protein that specifically binds to the death domain of the transmembrane death receptor Fas (also called CD95) in the cytoplasm and potentiates Fas-induced apoptosis. Over expression of Daxx enhances Fas-mediated apoptosis and activates the Jun N-terminal kinase (JNK) pathway. Daxx was found in the nuclears where it localizes to promyelocytic leukaemia (PML) oncogenic domains (PODs). As a highly conserved nuclear protein, Daxx plays an important role in proapoptosis, antiapoptosis and transcriptional regulation. This article reviews the latest advances in the study of the biological function of Daxx.

Key words: Death domain-associated protein (Daxx), cell apoptosis, transcriptional regulation, biological function.

INTRODUCTION

As a highly conserved nuclear protein, Death domain-associated protein (Daxx) was originally identified as a protein that specifically binds to the death domain of the transmembrane, death receptor FAS (also called CD95) in the cytoplasm and potentiates FAS-induced apoptosis (Yang et al., 1997). A large proportion of Daxx molecules are nuclear, however, associate with the promyelocytic leukaemia (PML) nuclear body (PML-NB) and other subnuclear domains. Daxx has been suggested to function as a pro-apoptotic protein downstream of FAS through activation of the c-Jun-N-terminal kinase (JNK) pathway in a FADD-independent manner (Yang et al., 1997). As a transcriptional regulator, Daxx regulates the transcription of a variety of factors and expresses different biological effects.

In recent years, both domestic and foreign researchers took a lot of research and explorations of Daxx, which were about apoptosis, anti-apoptosis and transcriptional regulation. This paper reviews the latest advances in the study of the biological function of Daxx.

THE BIOLOGICAL CHARACTERISTICS OF DAXX

Daxx is a highly conserved protein. Human Daxx protein is composed by 740 amino acids and its molecular weight is 81.4 KD. Mouse Daxx protein is composed of 739 amino acids, and its molecular weight is 81.3 kD. Daxx between the human and murine proteins has 69% identity. The total length of Daxx gene is about 315 kb and it has 7 exons and 6 introns. Because of the different post-transcriptional modification, Daxx molecules have three forms of existence, their molecular weight were 70, 97 and 120 ku, respectively. Daxx contains four regions,

*Corresponding author. E-mail: qhtuo@yahoo.com.cn.

which are two N-terminal paired amphipathic helices (PAHs); a coiled-coil domain; an acidic region and a C-terminal serine/proline/threonine rich region. These structure domains make close association with transcription of Daxx.

Daxx is ubiquitously expressed throughout the body, such as liver, kidney, heart, 293 cells, HeLa cell, etc. In addition, Daxx is also expressed in chronic lymphocytic leukemia (CLL) (Alkan et al., 2005).

DAXX AND PROAPOPTOSIS

Daxx was initially identified as a protein associated with the death domain of the Fas receptor and involved in Fas-mediated apoptosis as well as the activation of the JNK pathway. However, Daxx-mediated apoptosis signaling pathway, at present, is still not entirely clear. Confirming the initial yeast two-hybrid screen, glutathione S-transferase (GST) pull-down experiments showed the C-terminal portion of Daxx as interacting with the Fas receptor (YANG et al., 1997).

In order to further investigate the mechanism of Daxx, yeast two-hybrid technique was used to screen the intracellular proteins interacting with Daxx (Liu et al., 2012), and 13 positive colonies and three proteins interacting with Daxx were obtained. One of the candidate proteins was identified as ferritin, heavy polypeptide 1 (FTH1). The interaction between Daxx and FTH1 was further supported by GST pull-down and co-immunoprecipitation, respectively. Then, Daxx was determined to induce apoptosis through the Fas-Daxx-ASK1-JNK1 signaling pathway. Schepers et al. (2005) found out that an inhibitory role of HSP27 in VP-16-mediated activation of the ASK1/p38/JNK pathway after HSP27 combine with Daxx, which is in the monocytic acute myeloblastic leukemia (AML). As a highly conserved nuclear protein, Daxx deletion mutant (aa 501 to 625) has been known to be an inducer of apoptosis, and Song et al. (2004) observed that the Bax-dependent mitochondrial death signaling pathway plays an important role in Daxx 501 to 625-induced apoptosis. What is more, Daxx 501 to 625-induced apoptosis is mediated through the ASK1-MEK-JNK/p38-Bim-Bax-dependent caspase pathway.

Muromoto et al. (2010) found out that knock down of Daxx attenuated lactate dehydrogenase leakage from cells, indicating that Daxx positively regulates cell death during gp130/STAT3-mediated cell proliferation.

In exploring whether Daxx mediates oxidized low-density lipoprotein (Ox-LDL)-induced apoptosis in macrophage, He et al. (2010) found out that Ox-LDL up-regulated the expression of Daxx mRNA and caveolin-1, increased the accumulation of intercellular cholesterol and induced the apoptosis of RAW264.7 macrophages. However, Ox-LDL-induced intercellular cholesterol accumulation and apoptosis in RAW264.7 cells were prevented by Daxx siRNA. Ox-LDL also induced caveolin-1 expression and this effect was significantly suppressed by Daxx siRNA. It can be concluded that Daxx mediates Ox-LDL-induced cholesterol accumulation and apoptosis in macrophages by up-regulating caveolin-1 expression. We found out that over-expression of Daxx facilitated HepG2 cells apoptosis induced by hydrogen peroxide (Tuo et al., 2008). Furthermore, there may be a synergetic relation with apoptosis and increase of JNK activity. In order to elucidate the functional relevance of Daxx in apoptosis signaling of malignant lymphocytes, Jurkat T-cells were stably transfected with a Daxx-expressing vector or with the respective Daxx-negative control vector. Boehrer et al. (2005) demonstrated that ectopic expression of Daxx substantially increased the rate of apoptosis upon incubation with death receptor agonists such as Fas and TRAIL, as well as upon incubation with the cytotoxic drug doxorubicin (DOX). Although, enforced apoptosis caused by ectopic Daxx expression is caspase-dependent in both cases, major differences between Fas/Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-induced apoptosis and doxorubicin-induced apoptosis are observed in the expression patterns of X-linked inhibitor of apoptosis (XIA-P), p53, Bid, ZIP kinase and prostate apoptosis response gene 4 (Par-4). They outlined the major molecular mechanisms underlying the apoptosis-promoting effect of Daxx in neoplastic lymphocytes and demonstrated fundamental molecular differences elicited by the overexpression of Daxx, in the extrinsic and intrinsic signaling pathways. Sodium hydrogen exchange isoform 1 (NHE1) (Jung et al., 2008) was identified as a Daxx-interacting protein. During ischemic stress, Daxx translocates from the nucleus to the cytoplasm, where it colocalizes with NHE1. Daxx binds to the ezrin/radixin/moesin-interacting domain of NHE1, in competition with ezrin.

As most studies on the role of Daxx in cell death have been conducted using tumour cell lines, Khelifi et al. (2005) analyzed the function of Daxx in physiological settings. They employed RNA interference to down regulate Daxx in primary fibroblasts. Remarkably, Daxx-depleted cells are resistant to cell death induced by both ultraviolet (UV) irradiation and oxidative stress. Furthermore, the down regulation of Daxx results in impaired MKK/JNK activation. This is the evidence that Daxx promotes cell death and JNK activation in physiological conditions.

DAXX AND ANTIAPOPTOSIS

Despite the reports advocating a pro-cell-death function for Daxx, several studies have suggested a potential anti-apoptotic function for Daxx.

Michaelson and Leder. (2003) found out that Daxx was essential to the embryonic development and to be anti-apoptotic, based on the result those large quantities of embryonic stem cells apoptosis when Daxx gene was knocked out from the mouse embryo. The HeLa cell

sensitize to apoptosis in Daxx-knockout HeLa cell. Daxx can exert the anti-apoptotic function through regulating the two targets in Daxx, NF κB and E2F1.

Chen and Chen (2003) investigated the role of Daxx in Fas- and stress-induced apoptosis by small interfering RNA-mediated Daxx silencing in mammalian cells, and found out that Daxx silences strongly sensitized cells to Fas- and stress-induced apoptosis. These data strongly suggest that Daxx may inhibit Fas and stress-mediated apoptosis by suppressing pro-apoptotic gene expression outside of PML domains. Chaudhary et al. (2010) demonstrated that 4-hydroxynonenal (HNE) modification induced phosphorylation of Daxx at Ser668 and Ser671 to facilitate its cytoplasmic export, which consistently, induced apoptosis in HepG2 cells. However, the use of small interfering RNA-mediated Daxx silencing could induce apoptosis in HepG2 cells, these aspects indicated a potential antiapoptotic function for Daxx. Cermák et al. (2002) showed that over expression of Daxx inhibited anti-CD43 monoclonal antibody (mAb) MEM-59-induce apoptosis of the myeloid progenitor-derived cell line TF-1. Zhang et al. (2010) discovered that down-regulation of MDM2 in acute lymphocytic leukemia cells by berberine occurred at a posttranslational level through modulation of Daxx, which disrupted the MDM2–DAXX–HAUSP interactions and thereby promoted MDM2 self-ubiquitination and degradation. These results suggest a potential anti-apoptotic function for Daxx.

In human cells, Daxx interacted with heat shock factor 1 (HSF1) essentially only during stress, that is, when factor trimerization occurred. Daxx is an important mediator of HSF1 activation. Boellmann et al. (2004) suggest a model in which Daxx released from its nuclear stores during stress, opposes repression of HSF1 transactivation competence by multichaperone complex through its interaction with trimerized HSF1. Over expressed HSF1, known to be largely trimeric, only marginally increased HSF1 activity on its own, but potentiated the activating effect of Daxx overexpression.

Zobalova et al. (2008) found out that exposure of cultured cardiomyocyte-like cells to oxidative stress or simulated hypoxia increased the level of accumulated reactive oxygen species (ROS) and apoptosis. Under the conditions of sub-apoptotic stimulation of cardiac myocytes, there was no increase in the level of the Daxx protein, but its translocation from the nucleus to the cytoplasm was increased. Daxx over expression protect the cells from apoptosis, while they are sensitized to cell death following its down-regulation by siRNA. Moreover, lowering the level of the Daxx protein sensitized cardiac myocytes to spontaneous apoptosis, suggesting that the protein may also have a pro-survival role under physiological conditions. Daxx plays a protective role in cultured cardiomyocyte-like cells, at least under the conditions used.

The small ubiquitin-related modifiel-1 (SUMO)-1 is an important posttranslational regulator of different signaling pathways and is involved in the formation of promyelocytic leukemia (PML) protein nuclear bodies (NBs). Meinecke et al. (2007) showed that the increased expression of SUMO-1 in rheumatoid arthritis (RA) synovial fibroblasts (SFs) contributes to the resistance of these cells against Fas-induced apoptosis through increased sumoylation of nuclear PML protein and increased recruitment of the transcriptional repressor Daxx to PML NBs.

DAXX AS A TRANSCRIPTIONAL REGULATOR

Daxx has been shown to function as a transcriptional regulator via phosphorylation or its interaction with various cytoplasmic and nuclear proteins. Daxx is unlocated in PODs in the absence of PML or under normal circumstances, a condition in which PODs are destroyed. Daxx can localize to regions of condensed chromatin, when Daxx interacts with several transcription factors, including Pax3 (Lehembre et al., 2001), ETS1 (La et al., 2004). Daxx can inhibit their transcription activity, thus gene transcription is repressed and the cell apoptosis does not occur. In B cell lines, Daxx binding to transcription factor Pax5 and activating transcription of B cells shows the role of transcriptional co-activator. Park et al. (2007) found out that physical interaction between Daxx and p65 provides a functional framework for the inhibition of p65 acetylation by p300/CBP and subsequent repression of NF-κB transcriptional activity.

Daxx functions as a negative AR co-regulator through direct protein-protein interactions. Studies by Lin et al. (2004) revealed that the binding between Daxx and the DNA-binding domain of the ubiquitination AR shows marked suppression on the activity of the AR DNA-binding both *in vitro* and *in vivo*. Moreover, ubiquitination of AR at its amino-terminal domain is involved in Daxx interaction and trans-repression.

Chang et al. (2005) observed that Daxx suppresses Smad4-mediated transcriptional activity by direct interaction with the ubiquitination Smad4 and identified a novel role of Daxx in regulating TGF-β signaling pathway. Daxx also suppressed STAT3-mediated transcriptional activation, while over expression of Daxx inhibited IL-6/STAT3-mediated gene expression (Kenji et al., 2007). Boellmann et al. (2004) found that over expression of Daxx enhanced basal transcription activity of HSF1 in the condition of a stress. Zhang et al (2010) found out that the transcriptional inhibition of HSF4b is associated with Daxx. HSF4b can interact with Daxx in the nucleus and their association is regulated by the phosphorylation of HSF4b/S299. Thus, we can know the binding between Daxx and different transcription factor or related protein can influence the transcription.

Several recent studies have indicated that Daxx is a mediator of lymphocyte death and/or growth suppression, although, the detailed mechanism is unclear. Muromoto

et al. (2010) found out that Daxx suppresses the gp130/STAT3-dependent cell growth and that Daxx endogenously interacts with STAT3 and inhibits the DNA-binding activity of STAT3. Moreover, small-interfering RNA-mediated knockdown of Daxx enhanced the expression of STAT3-target genes and accelerated the STAT3-mediated cell cycle progression. In addition, knockdown of Daxx-attenuated lactate dehydrogenase leakage from cells, indicating that Daxx positively regulates cell death during gp130/STAT3-mediated cell proliferation.

CONCLUSION AND PROSPECTS

Currently, the precise function of Daxx in cell death and transcriptional regulation is not fully understood. While apoptosis appears to be an underlying theme in many of the Daxx studies, the role of Daxx in preventing and/or inducing apoptosis is as yet unclear. However, several studies had indicated that Daxx played significant roles in proapoptosis, antiapoptosis and transcriptional regulation. Certainly, the biochemical function and mechanism of Daxx are in progress and will be critical in further elucidating the functional role of Daxx. In addition, it will bring some novel ideas in prevent and tie at viral infection, cancer, leukemia and cardiovascular disease.

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Full Length Research Paper

Antifungal activity of essential oil from *Cinnamomum longepaniculatum* leaves against three dermatophytes *in vitro*

Cui Tao[1], Qin Wei[2], Zhong-Qiong Yin[1,2]*, Li-Jun Zhou[2], Ren-Yong Jia[1,2], Jiao Xu[1], Dong-Xia Shi[1], Yi Zhou[1], Yong-Hua Du[2] and Yun-Xia Deng[1]

[1]College of Veterinary Medicine Sichuan Agricultural University, Ya-an, 625014, China.
[2]Key Laboratory of Fermentation Resource and Application of Institutes of Higher Learning in Sichuan, Yibin College, Yibin 644000, China.

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The antifungal effects of essential oil from *Cinnamomum longepaniculatum* leaves were studied with special reference to minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC), time–kill studies and the mechanism of inhibition at ultrastructural level against *Trichophyton mentagrophytes*, *Microsporum canis* and *Trichophyton gypseum*. Its MICs against three important dermatophytes were 3.125, 3.125 and 3.125 ul/ml, while its MFCs against the same were 3.125, 3.125 and 3.125 ul/ml, respectively. All strains exposed to 1×MIC showed a maximum decrease in fungal inoculum of less than 2log10 CFU/ml within 12 h, and all the strains exhibited no growth within 72 h. Transmission electron microscope (TEM) of three important dermatophytes exposed to MIC levels of essential oil showed irreversible damage to cell wall, cell membrane and cellular organelles. The results are compared and with the data in literature. In conclusion, essential oil has significant antifungal activity *in vivo*, weakening the virulence of three dermatophytes and killing fungi by destroying the cell membrane and organelles.

Key words: Essential oils, antifungal activity, time-kill curve, transmitted electron microscopy (TEM).

INTRODUCTION

Cinnamomum longepaniculatum (Gamble) N. Chao, an endemic Lauraceae (Lauraceae) *Cinnamomum* plant species in China, has been listed as national key protected wild plant II level, which is mainly distributed in Sichuan Yibin, Hubei, Hunan, Shaanxi, Yunnan, Jiangxi, Guangxi, Guangdong and other places (Li et al., 1993; Tao et al., 2002). The chemical constituents of essential oils from *C. longepaniculatum* leaves found in Yibin of Sichuan Province in China have been studied by many researchers. Luo et al. (2001) analyzed the composition of the essential oils of the province. 26 main compounds were identified including 1,8-cineole (58.55%), abietinol, Sabinene etc. 1,8-cineole is used as food additives, often employed by the industry in drug formulations, as a percutaneous penetration enhancer and for its decongestant and pharmaceutical anti-tussive effects; it is used in aromatherapy as skin stimulant in the form of skin baths (Helander et al., 1998; Laude et al., 1994; Levison et al., 1994; Macht, 1938; Williams and Barry, 1991). Also, it is considered useful for the treatment of bronchitis, sinusitis and rheumatism (McGilevery and Reed, 1993). Wei et al. (2006, 2009) have demonstrated that essential oils from *C. longepaniculatum* leaves have antibacterial and antifungi toxic properties. But, the potential activities of the essential oils from *C. longepaniculatum* leaves against dermatophytes have not

*Corresponding author. E-mail: yinzhongq@163.com. Tel/fax: +86 835 2885614.

yet been evaluated. This study was undertaken to know the morphological changes of three important dermatophytes (*Trichophyton mentagrophytes, Microsporum canis* and *Trichophyton gypseum*) under restraint with essential oil; specifically, to find out the antimicrobial activity of essential oil from the leaves of *C. longepaniculatum* in Yibin. The results may be promising in controlling fungal growth and knowing the mode of action of antifungal components of *C. longepaniculatum*.

EXPERIMENTS

Materials

Essential oil, extracted from the leaf of *C. longepaniculatum* (Gamble) N. Chao using steam distillation, was purchased from a company in Yibin of China. It was kept at 4°C until it was used.

Microorganisms

The three dermatophytes used during the growth experiments are as follows: *Trichophyton mentagrophytes*, *Microsporum canis*, and *Trichophyton gypseum*, supplied by the Sichuan Animal Husbandry and Veterinary Institute, Sichuan, China. The samples of mycelium necessary for the *in vitro* experiments were taken from cultures grown in slants and kept on Sabouraud's agar (SDA). Spore suspensions were prepared and diluted in sterile Roswell Park Memorial Institute-1640 (RPMI-1640) broth to a concentration of approximately 10^7 CFU/ml. Spore population was counted using haemocytometer. Subsequent dilutions were made from theaforementioned suspensions, which were then used in the tests.

Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations determinations

MIC of the samples was determined by the broth dilution method using RPMI-1640 broth as described previously (Espinel-Ingroff et al., 2002; Trilles et al., 2004). The media containing 100 µl/ml of essential oils were serially diluted twofold each to give concentrations of 100, 50, 25, 12.5 6.25, 3.125, 1.563, 0.781 and 0.391µl/ml. To the diluted solution, equal volume of RPMI-1640 media inoculated with three dermatophytes at a concentration of 2×10^4 CFU/ml was added and mixed well. The mixture was incubated at 27°C for one week to check the minimal concentration, at which growth of the fungi cells was fully inhibited. The least concentration showing no visible growth on sterile sabouraud's agar (SDA) by subculture was taken as MFC value. A similar experiment was carried out using DMSO instead of essential oils from *C. longepaniculatum* leaves as a control. Three replicates were used for each concentration and all experiments were repeated twice.

Kill-curve studies

The effects of essential oil exposure in relation to time and concentration on three dermatophytes were determined in RPMI-1640 liquid medium. Conidial suspensions (1×10^3 to 1×10^4 CFU/ml) were incubated in the presence of MIC concentrations of essential oil from *C. longepaniculatum* leaves (3.125 µl/ml). 312.5 µl of essential oil at the dilution determined by MIC was added to 100 ml of each spore suspension containing 1×10^3 to 1×10^4 CFU/ml and was then incubated at an ambient atmosphere at 27°C in an incubator shaker. Samples were taken after 0, 2, 4, 8, 10, 12, 24, 36, 48, 60 and 72 h; and 0.1 ml of aliquot of the conidial suspension was removed and diluted to obtain 10^1 to 10^3 dilutions. 1 ml of aliquot was cultured on SDA plates and incubated in an ambient atmosphere at 27°C for one week and the total number of viable spores per ml was calculated. Kill–curves were constructed by plotting mean lgCFU/ml against the time of exposure of conidia to essential oil from *C. longepaniculatum* leaves.

Sample preparation for TEM

The essential oil from *C. longepaniculatum* leaves was added to a culture medium and reached a final concentration of 3.125 µl/ml with optical density of 10^6 CFU/ml; then it was incubated on a shaker at 27°C for 24 h. Mycelial samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate-buffer (pH =7.4) at 4°C overnight. They were washed three times, each time for 15 min, in cacodylate-buffer. Specimens were then post-fixed for 2 h in 1% osmium tetroxide (OsO4) dissolved in cacodylate-buffer at room temperature and washed in cacodylate-buffer (three times for 15 min each). Samples were dehydrated in a graded series of ethanol (40, 60, 75, 80, and 95%, two times for 15 min each and two times for 30 min each in 100% ethanol). Epoxy resin (Epon-618) was used to embed the post-fixed fungi samples for 12 to 16 h at 45°C. Ultra thin section of the embedded samples was prepared by LKB-II Ultracut instrument and double stained with uranyl acetate and lead citrate. The samples were lyophilized and then examined by transmitted electron microscope on a Hitachi TEM-1011 instrument. The optical density at 10^4 CFU/ml was recorded to evaluate the growth of the fungi.

RESULT AND DISCUSSION

MIC and MFC studies

The MICs and MFCs of the essential oil against three dermatophytes estimated by the microdilution technique were equally 3.125 µl/ml. It was considered that if essential oil once reached MIC concentration, it displayed fungicidal activity. The MICs and MFCs found for essential oil against pathogenic fungi plant were similar to the values of *in vitro* susceptibility reported in the literature (Wei et al., 2006).

Kill–curve studies

The effect of essential oil on the growth of three dermatophytes is illustrated in Figure 1. The results indicate that concentrations of 1×MIC of essential oil had an inhibitory effect on the growth of these organisms. Essential oil exerted maximal killing within 6 to 8 h in all experiments with a concentration of 1×MIC. All strains exposed to 1×MIC showed a maximum decrease in fungal inoculum of less than 2log10 CFU/ml within 12 h and all the strains exhibited no growth within 72 h (Figure 1). The changes in fungal inoculum of *M. canis* after exposure to an essential oil concentration of 1× MIC were comparable with those *of T. mentagrophytes* and *T. gypseum* (Figure 1). The results suggest a maximum killing ability against *Microsporum canis* at 1× MIC and that no further killing can be achieved against the other two strains at 1 × MIC.

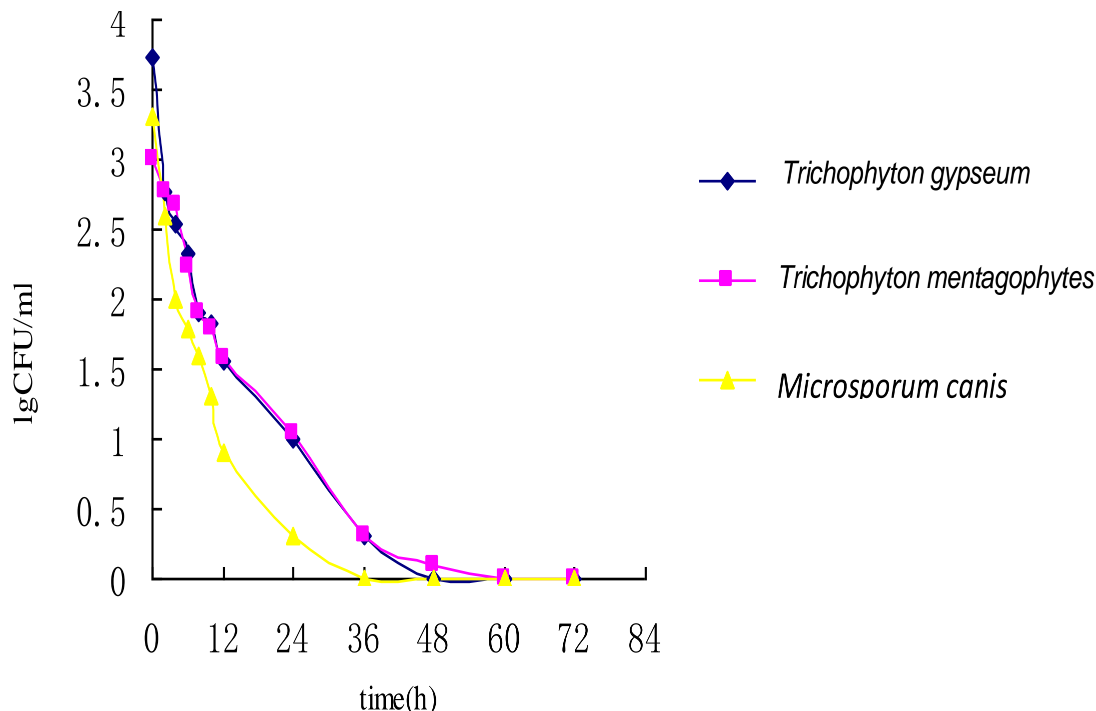

Figure 1. The time-kill cure of essential oil at MIC concentration from *C. longepaniculatum* leaves against three dermatophytes.

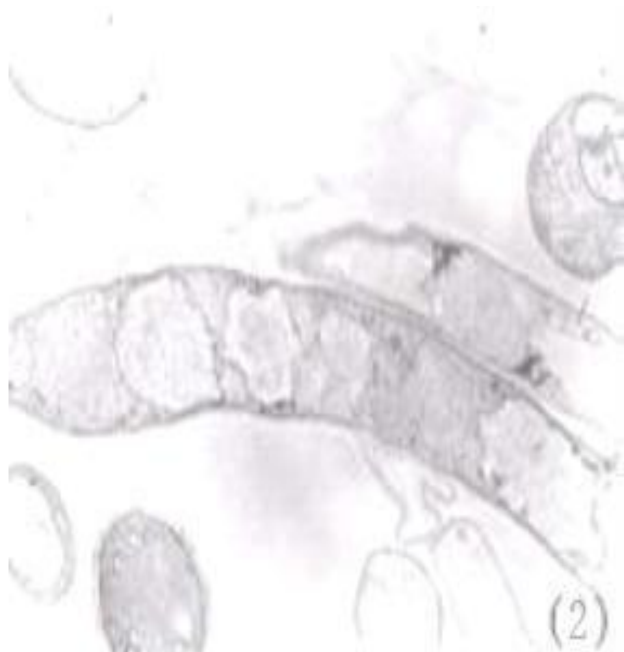

Figure 2. Ultrastructure of *T. mentagrophytes* were untreated with 1×MIC essential oil with normal septum, endomembrane system, wall and organelles (G×8,000).

Our most striking finding was that starting essential oilconcentrations of 1× MIC were largely effective and significantly inhibited all isolates; whereas the residence time of essential oil *in vitro* brought about by declining sterilizing ability. The reduction of sterilizing ability may be due partly to volatile effective constituents. The present study suggests that essential oils concentration of MIC yielded an excellent fungal effect on three dermatophytes and might be sufficient to achieve fungal killing over a 72 h period.

The result of TEM

Scanning electron micrographs of three dermatophytes treated or untreated with 1×MIC essential oil for 24 h are as follows:

1. Solvent controls samples (Figure 2): The ultra-structure of untreated *T. mentagrophytes* cell is shown in Figure 2. Mycelia were made up of long strands of hyphae, normally elongated with smooth walls where outer membrane, periplasm and cytoplasmic membrane could be seen.
2. The experimental group (Figure 3): *T. mentagrophytes* treated with essential oil are shown in (Figure 3). The fungal cells showed morphological changes; bleb-like structures, were observed. The ultrastructural changes included an alteration of the space between the cell wall and the plasma membrane, deformed cells, disorganization and depletion of membranous organelles and reduction

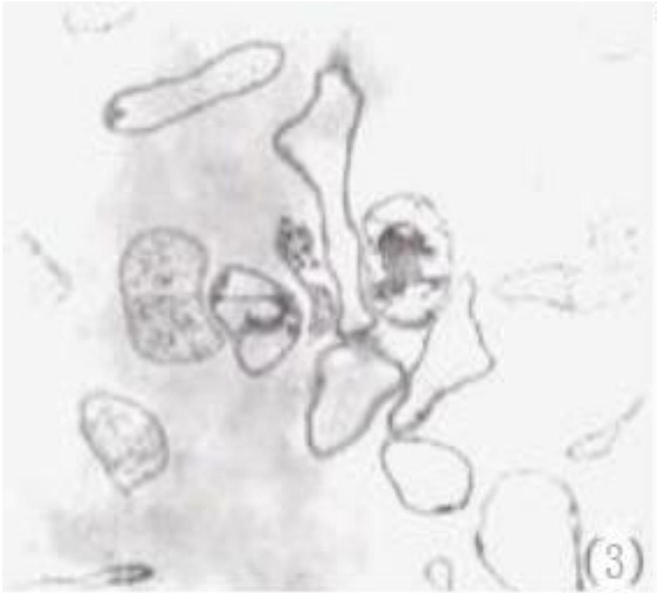

Figure 3. Ultrastructure of *T. mentagrophytes* were treated with 1×MIC essential oil with disorganization of cytoplasm and cytoplasm content, and losing its integrity.of outer wall(G×12,000).

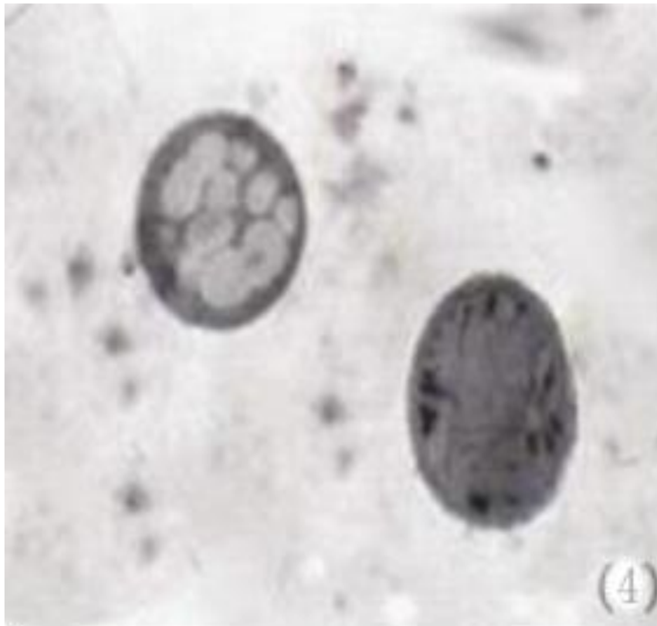

Figure 4. *M. canis* were homogenous. The cell wall, plasma membrane and intracellular organelles including mitochondria, vesicles, electron dense granules and nucleus had uniform and normal structures (G×12,000).

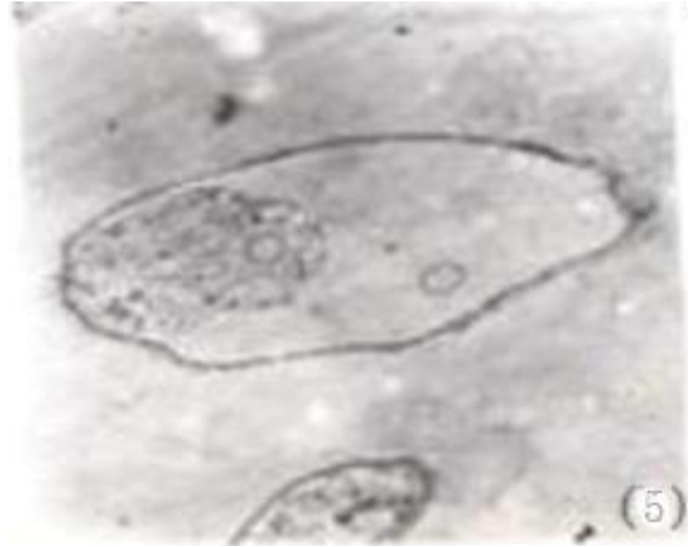

Figure 5. *M. canis* complete depression and disorganization signs including extensive destruction and lysis of hyphae membranous organelles such as nucleus and mitochondria, massive vacuolation of cytoplasm with vacuole fusion, complete autolysis and disorganization of hyphae cytoplasm (G×15,000).

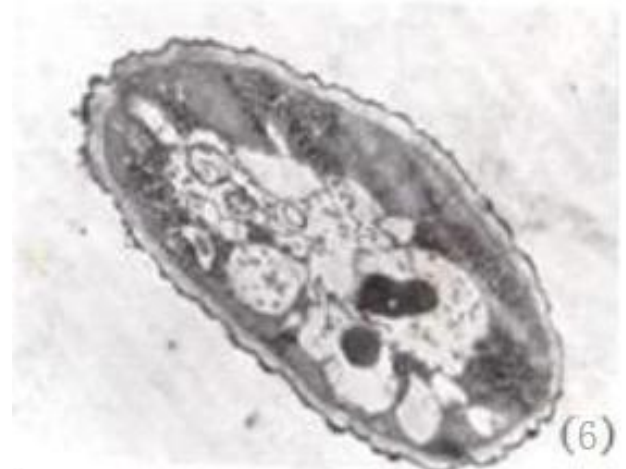

Figure 6. *T. gypseum* revealed typical eukaryotic cytoplasmic components including numerous ribosomes, mitochondria and vacuoles in the cytoplasm enclosed by an electron-transparent cell wall(G×6,000).

in cell size.

3. Solvent controls samples (Figure 4): *M. canis* maintained a normal morphology with membrane, periplasm, cytoplasmic membrane, nuclei, ribosomes and numerous granules of glycogen. The plasmalemma, adherent to the cellular wall and the innersystem of endomembrane showed a normal morphology.

4. The experimental group (Figure 5): The outer membrane of *M. canis* rose up and separated from the wall, or it may even be stripped away and dispersed in the outside medium. Disorganization signs included extensive destruction and lysis of hyphae membranous organelles.

5. Solvent controls samples (Figure 6): TEM observation of untreated *T. gypseum* hyphae revealed typical eukaryotic cytoplasmic components including numerous ribosomes, mitochondria and vacuoles in the cytoplasm

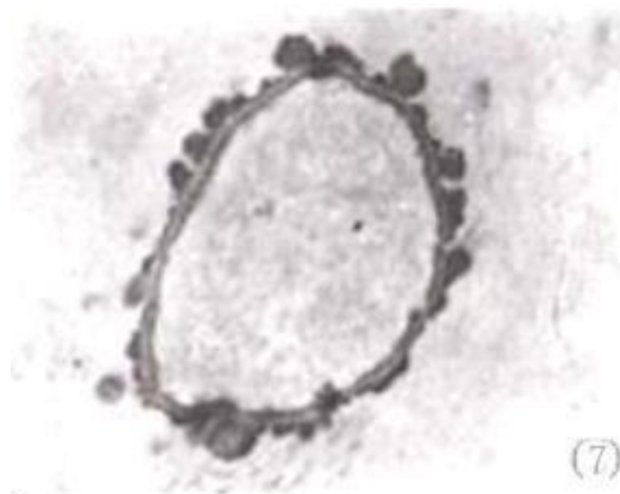

Figure 7. Normal *T. gypseum* with electron- transparent vacuoles, and deeply altered cells, with hardly recognizable organelles were visible (G×8,000).

antifungal activity of Chinese herbs, their mechanism of action is not well understood. Some literatures have reported (Ghahfarokhi et al., 2004; Jiang and Cao,1999; Moreira et al., 2005; Parka et al., 2009; Soylu et al., 2006) the mechanism of the antifungal activities of some Chinese herbs, such as damaging the integrity and continuity of fungal cell wall and cell membrane, causing cell swelling , necrotizing or disintegrating organelles, affecting its nucleic acid or lipid metabolism and so on. When treated with 1×MIC essential oil for 24 h, *T. mentagrophytes* showed shrunk hyphe cell, dissolved or destroyed organelle and nucleus. Meanwhile, the cyto-plasm of *M. canis* and *T. gypseum* was occupied by low electron-densed areas and generated large amounts of white space. The deleterious effect of the essential oil on the three dermatophytes may be the main reason for the highly hydrophobic components of essential oil, which may readily pass through the cellular membrane and change its permeability. This leads to organelles disintegration or collapse, decrease of electron density, formation of a cystic space, restrain on the growth of mycelia, formation and germination of sporules; and eventually the fungi decay and die.

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Full Length Research Paper

Effect of concentration on polarizability of urinary bladder membranes using potassium chloride solutions

P. C. Shukla* and Aalok Shukla

Bio-Physical Laboratory, Department of Chemistry, St. Andrews Post Graduate College, Gorakhpur (U. P.), India.

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Potassium and chloride ions are vital in human bodies. Medically it is used for the treatment of hypokalemia and digital poisoning. Gastro intestinal discomfort, diarrhea, vomiting etc are some of the side effects. Its overdose causes hyperkalemia which can cause cardiac block and continued increase or decrease of potassium and chloride ions may have adverse effect in the frequency of urination. With this end in view, hydrodynamic and electro-osmotic permeability of aqueous solution of potassium chloride have been carried out across urinary bladder membranes of goat. Kinetic energy term (α_1) which is equivalent to velocity head which decreases effective pressure of the membrane and polarizability term (α_2) which is related with distention power of the bladder have been calculated for different solution of potassium chloride solutions. It has been found that α_1 increases in concentration of potassium chloride while α_2 follows a reverse trend.

Key words: Polarizability, potassium, chloride ions, urinary bladder, membranes.

INTRODUCTION

Potassium and chloride ions are the essential constituents of urine (Oser, 1979). Osmotic pressure regulation and acid-base balance of the body are the primary functions of these ions (West and Told, 1974). Potassium is the major base of body cells and it has been shown to aid in enzymatic transfer of phosphate from adenosine-5'-triphosphate (ATP) to pyruvic acid. Potassium ion has an effect on muscular irritability. Maintenance of proper potassium ion concentration of extra cellular fluid is essential, particularly for proper function of the heart. High concentration of potassium ion cause wide spread intra cardiac block while low concentration impairs the contractility of heart muscle. Potassium may play (Lininger, 2000; Griffith, 2001; Sigworth, 2001) a role in prevention and treatment of diabetes, cataract, osteoporosis, high blood pressure and dehydration effects etc. Special significance lies in the fact that during fasting, however because of lack of NaCl intake and release of much more K^+ than Na^+ in the catabolism of tissue, more potassium than sodium is excreted in urine. The urinary excretion of potassium relative to sodium is increased in acute felirile conditions involving tissue destruction.

Urination is the collective property (Shukla, 1995; Chaube, 1994, 1995; Shukla and Mishra, 1987) of the bladder, urine and the forces responsible for it. Interaction with bladder surface plays vital role in micturition phenomena. With this end in view, role of potassium chloride (KCL) solutions in deciding polarizing power of bladder membranes have been studied in detail using methodology of non-equilibrium thermodynamics. Development of pressure, sustenance of pressure and finally release of pressure (Guyton, 1981) is the basis of micturition phenomena. Development of pressure produces streaming potential (He et al., 2005) which in turn gives rise to streaming current (Shukla and Mishra, 1987). This streaming current is nothing but micturition wave. The micturition reflex occurs on account of

*Corresponding author. E-mail: pcshuklabpl@yahoo.com.

micturition waves and is self regenerative. In other words, polirizing power of the bladder decreases with increase in concentration of potassium chloride. Thus frequency of urination is affected by increase of potassium chloride across urinary bladder membranes.

THEORETICAL

Since urination process is the collective property of pressure and electrical potential gradient, volume flow may be expressed as (Lakshminiarh, 1984, Rastogi and Singh 1963).

$$J_v = L_{11}\Delta P + L_{12}\Delta\psi + 1/2\ L_{111}(\Delta P)^2 + L_{112}\,\Delta P\,\Delta\psi + 1/2\ L_{122}(\Delta\psi)^2 + 1/2\ L_{1112}(\Delta P)^2\Delta\psi + 1/2\ L_{1112}(\Delta P)(\Delta\psi)^2 + 1/6\ L_{1111}(\Delta P)^3 + \ldots\ldots$$

where L_{ij}, L_{ijk}, L_{ijkl} (k, l = 1, 2) are phenomenological coefficient.

Kinetic energy term (α_1) and polarizability term (α_2) may be expressed as (Lorimer, 1985).

$$\alpha_1 = \frac{L_{111}A^2}{\rho\, L_{113}} = -\left[\frac{L_{1111}\,A^4}{3\,\rho^2 L_{11}^{\ 5}}\right]^{1/2}$$

$$\alpha_2 = -\frac{L_{1222}A^2}{3\rho\, L_{11}^{\ 2} L_{12}\,\alpha_1} - \frac{\alpha_1\,\rho\, L_{12}^{\ 2}}{2A^2} - \frac{L_{1122}A^2}{\alpha_1\rho\, L_{11}^{\ 3}} - \frac{3\,\alpha_1\,\rho\, L_1}{A^2}$$

where L_{1222} and L_{1122} are higher order phenomenological coefficients.

MATERIALS AND METHODS

The membrane chosen for the experimental study is the urinary bladder of goat. It is a smooth muscle chamber having different layers (Bailey et al., 1979). It was chosen due to easy availability and capability to stand high pressure. The membrane was isolated from the animal and then immediately dipped in 0.7% sodium chloride solution. Care was taken to see that urinary bladder contained some urine. After keeping the urinary bladder for 2 to 3 h, it was treated with formalin alcohol (100 parts water, 125 parts 95% alcohol and 10 parts 40% formaldehyde) solution (Shukla and Mishra, 1986). The membrane was then reheated and washed with double distilled water and maintained in a wet state by filling it up with aqueous urea solution (≈ 0.01 M) across the membrane in the experimental setup. Hydrodynamic permeability was measured by applying pressure across one side of the membrane with no volume charges in the horizontal capillary. Electro-osmotic (Shukla and Mishra, 1987) permeability was measured by applying electrical potential difference across one side of the membrane with no volume charges across the membrane.

RESULT AND DISCUSSION

Potassium is the major base of body cells. Potassium ion has an effect on muscular irritability. Potassium deficiency in man is associated with weakness and muscular paralysis. In Addison's disease, the excretion of potassium falls and plasma potassium rises, with reverse changes in urinary and plasma sodium indicating a function of adrenal cortex in this connection. Potassium is largely excreted by the kidney, although the mechanism is unsettled.

The excretion of chlorides in urine is decreased when blood chloride levels are lowered by losses through diarrhea and excessive vomiting. Urine chlorides may be extremely low in case of severe diabetes insipidus. Control of K^+ balance of the body by the kidney is not as efficient as control of Na^+. Efficient diet may show practically no Na^+ excretion as there is always an obligetory excretion of potassium, regardless of potassium intake which continues even during prolonged starvation with the K^+ arising from cellular breakdown. Electrolyte disturbances can interfere with membrane permeability (Murray et al., 2000). Beside these, potasium ion participates in a number of physiological process including maintenance of intracellular tonicity, transmission of nerve impulse, contraction of cardiac, skeletal and smooth muscles and the maintenance of normal renal function. It has been observed that the alkalizing salt, $KHCO_3$ is a strong and neutral salt; KCl is a weak promoter of urinary bladder carcinogenesis in rat (Lina et al., 1994).

The role of potassium absorption in normal bladder has been correlated with sensory urgency (Parson et al., 1998). Chronic low grade metabolic acidosis occurs in various renal disorders. In normal people it is related both to dietary net acid load and age related functional decline and may contribute to osteoprosis by increasing urine calcium excretion (Fressetto et al., 2000). The effect of short term supplementation of KCl and potassium citrate on bladder pressure in hyper sensitivities gives useful information (He et al., 2005). The significance of KCl sensitive test is of prime importance in urological problems (Alp et al., 2007). Results suggest that high KCL supplementation increases water intake, urine volume and urinary nitrogen excretion in mice (Murari et

Table 1. Values of phenomenological co-efficient.

SL. No.	Concentration (mol/L)	$L_{11} \times 10^{-3}$ $m^5s^{-1}N^{-1}$	$L_{12} \times 10^{-11}$ $m^3s^{-1}N^{-1}v^{-1}$	$L_{111} \times 10^{-14}$ $m^7s^{-1}N^{-2}$	$L_{122} \times 10^{-11}$ $m^3s^{-1}N^{-1}v^{-2}$	$L_{1222} \times 10^{-13}$ $m^3s^{-1}N^{-1}v^{-3}$	$\alpha_1 \times 10^{16}$ $m^{-1}s^{-1}Ns^{2-1}kg$	$\alpha_2 \times 10^3$
1	0.02	0.760	1.90	4.25	0.140	2.44	0.96	-7.30
2	0.04	0.725	1.60	4.65	0.125	3.05	1.22	-10.57
3	0.06	0.685	1.36	4.75	0.100	3.60	1.52	-14.27
4	0.08	0.635	1.05	5.00	0.085	4.25	1.89	-15.87
5	0.10	0.625	0.64	8.35	0.040	4.80	3.55	-17.59

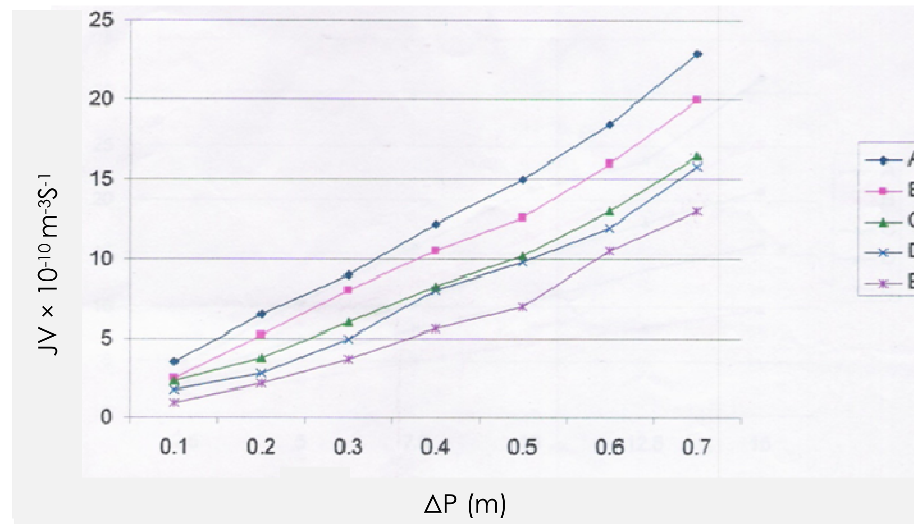

Figure 1. Dependence of volume flux against pressure difference for KCl solution.

al., 2010). Proper excretion of urine from the bladder is a clinical index of renal function. Generation of voiding tendency by the bladder depends upon proper interaction of bladder, urine and the forces responsible for urination.

Micturition reflex is a single complete cycle of: (i) progressive and rapid increase of pressure, (ii) a period of sustained pressure and (iii) return of pressure to the basal tonic pressure of the bladder.

Development of pressure gives rise to streaming potential which in turn produces streaming current. This streaming current is probably responsible for micturition waves and finally micturition reflex. As soon as micturition takes place, pressure build up is almost zero. The streaming potential and current tend to be minimum. Again, with the filling of the bladder, pressure build up produces streaming potential and current. This process is continuous.

Since the experiment on streaming potential

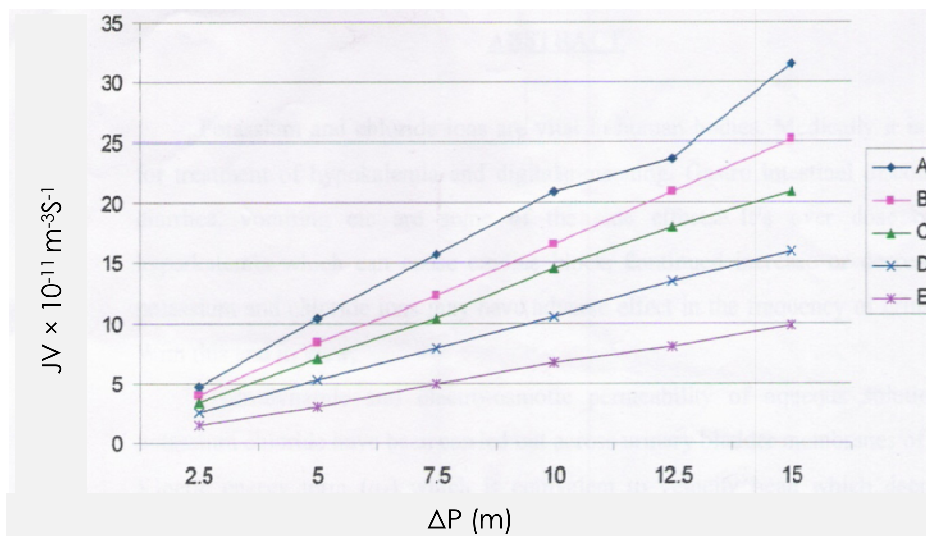

Figure 2. Dependence of volume flux against electrical potential difference for KCl solution.

in concentration as shown in Figure 2

(3) α_1 increases with increase in concentration of KCl

(4) α_2 decreases with increase in concentration.

Values of higher order phenomenological coeffiecients (Table 1) are evaluated using extra population technique (Shukla and Mishra, 1987).

Kinetic energy (α_1) which is equivalent to velocity head decreases effective pressure across the membrane and polarizability term (α_2) is related with distention power of the bladder. Increasing value of α_1 and decreasing value of α_2 gives the same result. In this case, same trend is observed. Thus, frequency of urination may be affected by increase of KCl in urine which may be of immense physiological importance.

Symptoms of trouble in urine excretion, hindrance to urinate in the morning and trouble of micturition have been reduced by ethno-medicines and discussed in detail recently (Naumi Emmanue, 2010).

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Full Length Research Paper

Antifungal activity and gas chromatography coupled with mass spectrometry (GC-MS) leaf oil analysis of essential oils extracted from *Eucalyptus globulus* (Myrtaceae) of north centre region of Morocco

Elhoussine derwich[1]*, Zineb Benziane[2], Rachida Chabir[3] and Amina Bouseta[2]

[1]Unity of GC/MS and GC, Regional Center of Interface, University Sidi Mohamed Ben Abdellah, BP, 2626, Rouad Immouzer, Fez, Morocco.
[2]Department of Biology, Faculty of Sciences, University Sidi Mohamed Ben Abdellah, Fez, Morocco.
[3]Department of Biology, Faculty of Medicine and Pharmacy, University Sidi Mohamed Ben Abdellah, Fez, Morocco.

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Essential oil from the leaves of *Eucalyptus globulus* collected in north centre region of Morocco obtained by hydro-distillation were analyzed by gas chromatography equipped with flame ionisation detector (GC-FID) and gas chromatography coupled with mass spectrometry (GC-MS). To evaluate the antifungal activities of these aromatic extracts, their *in vitro* antifungal activities were determined by disk diffusion testing to find out minimum inhibitory concentration (MIC). *Penicillium citrinum* was used as test fungal strains. The results of the study revealed that essential oil yields and the total oil of *E. globulus* were 1.21 and 63.96%, respectively. 54 compounds were identified in the essential oils and the main constituents of the essential oils were: 1.8-cineole (22.35%), limonene, (7.01%), solanol (6.05%), β-pinene (5.20%), trans-verbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinene (1.49%), α-myrcene (1.15%) and α-terpineol (1.10%). The essential oil of *E. globulus* exhibited the activity against, *P. citrinum* exerting the minimum inhibitory concentration values (MIC) ranging from 3.07 to 96.14 µl/ml, respectively. These results showed that extracts could be considered as a natural antifungal source that can be used for production of natural antifungal agents.

Key words*:* *Eucalyptus globulus*, essential oil, gas chromatography coupled with mass spectrometry (GC-MS), antifungal activity, 1.8-cineole.

INTRODUCTION

The essential oils which were utilised centuries ago in cosmetics usually show interesting biological features. Essential oils were used in ancient Rome, Greece and Egypt and throughout the Middle and Far East as perfumes, food flavours, deodorants and pharmaceuticals (Baris et al., 2006). Medicinal plants have been used as a source of remedies since ancient times and the ancient Egyptians were familiar with many medicinal herbs and were aware of their usefulness in treatment of various diseases (Abu-Shanab et al., 2004). Until recently, essential oils have been studied most from the viewpoint of their flavour and fragrance chemistry only for flavouring foods, drinks and other goods. Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey et al., 2001; Gianni et al., 2005; Sawamura,

*Corresponding author. E-mail: elhoussinederwich@yahoo.fr. Tel: 00. 212. 457. 56. 052.

2000). Plant essential oils and their components have been known to exhibit biological activities, especially antimicrobial, since ancient time. With the growing interest in the use of either essential oils or plant extracts in the food and pharmaceutical industries, screening of plant extracts for these properties is of increasing importance (Amvam et al., 1998). The World Health Organization has recommended and encouraged the use of chewing sticks (Almas and Al Lafi, 1995). *Eucalyptus* belongs to the family Myrtaceae, and is a globally distributed genus important as one of the two most-extensively planted pulpwood plantation species (Zobel, 1988). Many species of the genus *Eucalyptus* are used in many parts of the world for the treatment of a wide variety of diseases including microbial infections (Benarfa et al., 2007). Several studies have been reported on the chemical composition of the essential oils of *Eucalyptus* species belonging to different regions in the world (Benayache et al., 2007; Chalchat et al., 1997; Menut et al., 1992).

Morocco is blessed with a rich source of aromatic plants, many of which have not been previously investigated for their chemical constituents and biological potentials. *Eucalyptus globulus* is a plant that belongs to the family Myraceae, which grows in Morocco region and is a potential source of essential oils

The aim of this study was to elucidate the chemical constituents and evaluate the antifungal activity of the essential oil of the leaves of *E. globulus* collected in Atlas mean (Tichoukt), a mountainous region of Morocco.

MATERIALS AND METHODS

Chemicals and standards

All solvent were of analytical grade, unless otherwise specified. Hexane solution, anhydrous sodium sulfate, fungal strains**,** series of alkanes (C_4 - C_{28}) standards were obtained from, Faculty of Sciences, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

Plant material

The leaves of *E. globulus* were collected in April 2010 at Skoura (Tichoukt) near Boulmane, 90 km in the south east of Fez. The climate is semi-humid with strong continental influence with an annual average temperature of 20°C. The plants were then isolated from the other specimen and conserved for extraction.

Essential oil extraction

The leaves of *E. globulus* were shade dried (25 days) at room temperature and immediately hydro-distilled (500 g) for 3.5 h using a modified Clevenger-type apparatus. The oil was extracted from the distillate with hexane and then dried over anhydrous sodium sulfate. After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 35°C and the pure oil kept at 4°C in the dark, until the moment of analysis.

Gas chromatography analysis (GC/FID)

The quantitative analysis of essential oil of *E. globulus* was done with the help of a chromatographer in gas phase equipped with flame ionisation detector (GC-FID, Trace GC ULTRA S/N 20062969, Thermo Fischer), Varian capillary column (5% poly diphenyl 95% dimethylsiloxane, TR5- CPSIL- 5CB; 50 m length, 0.32 mm of diameter and film thickness 1.25 µm). The column temperature was programmed from 40 to 280°C for 5°C/min and finally held at that temperature for 10 min. The temperature of the injector was fixed to 250°C and the one of the detector (FID) to 260°C. The debit of gas vector (azoth) was fixed to 1 ml/min and split injection with split ratio 1:40. The volume injected was 1 µl of diluted oil in hexane solution (10%). The percentage of each constituent in the oil was determined by area peaks.

Gas chromatography-mass spectrometry

The identification of different chemical constituents was done by gas phase chromatography (Ultra GC Trace) coupled with spectrometer (PolarisQ/ S/N 210729, Thermo Fischer); with ionisation energy of 70 ev. The utilised column was; Varian capillary column (TR5- CPSIL- 5CB; 50 m length, 0.32 mm of diameter and film thickness 1.25 µm). The column temperature was programmed from 40 to 280°C for 3°C/min. The temperature of the injector was fixed to 260°C and the one of the detector (PolarisQ) to 200°C. The debit of gas vector (Helium) was fixed to 1 ml/min. The volume of injected specimen was 1 µl of diluted oil in hexane. The constituents of essential oils were identified in comparison with their Kovats index, calculated in relation to the retention time of a series of lineary alkanes (C_4- C_{28}) with those of reference products and in comparison with their Kovats index with those of the chemical constituents gathered by Adams (2001) and in comparison with their spectres of mass with those gathered in a library of (NIST-MS) type and with those reported in the literature (Derwich et al., 2011).

Antifungal assay

In the last few years, there has been target interest in biologically active compounds, isolated from plant species for the elimination of pathogenic microorganisms, because of the resistance that microorganisms have built against antibiotics (Essawi and Srour, 2000) or because they are ecologically safe compounds (Lee et al., 2005). The antifungal activity of the extracts from *E. globulus* was measured according to the procedure described by Adiguzel et al. (2002) with some modifications.

The dried plant extracts were dissolved in the same solvent (ethanol) to a final concentration of 30 mg/ml and sterilised by filtration through 0.45 µm Millipore filters. Antifungal tests were then carried out by the disc diffusion method (Murray et al., 1995) using 50 µl of suspension containing 52 spore/ml of fungi spread on nutrient agar (NA), sabouraud dextrose agar (SDA), and potato dextrose agar (PDA) mediums, respectively. The discs (6 mm in diameter) were impregnated with 10 µl of essential oil or 30 mg/ml extracts (300 µg/disc) placed on the inoculated agar. Negative controls were prepared using the same solvents as that employed to dissolve the plant extracts. Ofloxacin (10 µg per disc), sulbactam (30 µg)+ cefoperazona (75 µg) (105 µg/disc) and/or netilmicin (30 µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 27 for 72 h with fungi isolates. Antifungal activity was evaluated by measuring the zone of inhibition against the test organisms and the minimal inhibition concentration (MIC) values were evaluated according to published procedures (Celikel and Kavas, 2008; Bounatirou et al., 2007; Baratta et al, 1998). Each assay in this experiment was repeated twice.

RESULTS AND DISCUSSION

Phytochemical content of the leaf essential oils

Results obtained for the yields, compositions, contents, and identification of the leaf essential oils of *E. Globules* oils have been shown in Table 1. Yields of leaf essential oils from the hydro-distillation of *E. globulus* were 1.21%. In this study of the leaf essential oil of *E. globulus*, 54 compounds were identified, which made up 63.96% of the total essential oil and the major constituents was: 1.8-cineole (22.35%), other components present in appreciable contents were limonene, (7.01%), solanol (6.05%), β-pinene (5.20%), trans-verbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinène (1.49%), α-myrcène (1.15%) and α-terpinéol (1.10%).

The chemical compositions of the leaf oils of Eucalyptus from various parts of the world have been reported. 1.8-Cineole was identified as the major component in samples growing in Taiwan (Yu-Chang et al., 2006), Uruguay (Dellacassa et al., 1990), Algeria (Benayache et al., 2001), Burundi (Dethier et al., 1994), Congo (Cimanga et al., 2002), Mozambique (Pagula et al., 2000), Greece (Tsiri et al., 2003), Australia (Brophy et al., 1991), Tunisia (Bendaoud et al., 2009), Italy (Gianni et al., 2005), Nigeria (Islaka et al., 2003) and Turkey (Azcan et al., 1995). Also, 1.8-cineole was identified as the major component in others plants: *Laurus Nobilis* (Derwich et al., 2009; Ozcan and Chalchat, 2005); *Origanum minutiflorum* (Dadalioglu and Evrendilek, 2004); *Eucalyptus smitii* and *Callistemon speicosus* (Ntezurubanza, 2000). Previous studies of the leaf oil compositions of *Eucalyptus* species used commercially as a natural source of 1.8- cineole have been reported (Dethier et al., 1994; Boland et al., 1991).

The essential oils composition obtained in this study showed a relatively similar pattern to those published for other geographical regions: 1.8-cineole (84.7%), α-pinene (4.4%), trans-pinocarveol (2.2%), were reported as the major component in the essential oil of *Eucalyptus viridis* and 1,8-cineole (89.4%), β-pinene (1.2%) and α-pinene (1%) of *Eucalyptus oleosa* from Iran (Jaimand et al., 2009), oxygenated monoterpene: 1,8-Cineole (69.53%) and the monoterpene hydrocarbon: α-pinene (11.94%) from Tunisia (Bendaoud et al., 2009). Also it is different to the chemical composition of essential oil of leaves of *Eucalyptus robusta* and *Eucalyptus saligna* reported in Brazilian study in which the major component were *α*-pinene (73.0%) and *p*-cymene (54.2%) respectively (Patrícia et al., 2007) and they are different to those found in *Eucalyptus tessellaris* oil in Australia (Bignell et al., 1997) and Nigeria (Siaka et al., 2005), in which the major component was α-pinene (0.1-64.4%) and (46.60%) respectively,. Intense studies on Genus *Eucalyptus* essential oil composition have been published already (Nair et al., 2008; Gamal et al., 2007).

In this study, the yields of the oils obtained from the hydro-distillation of the leaves of *E. globulus* was 1.21%; it is relatively lower than other plants as a source of essential oils: *Myrtus communis* (1.75%) (Derwich et al., 2011), (*Eucalyptus microtheca* (2.3%), *Eucalyptus tereticornis* (3.4%) and *Eucalyptus grandis* (4.7%) (Islaka et al., 2003) and it is higher to the yield of essential oil isolated by hydro-distillation of the needles with twigs of *Pseudosuga menziesii* which was found to be 0.67% based on fresh material (Tesevici et al., 2009). Also it is higher to the yield of essential oil extracted of *Mentha piperita* from Morocco by Derwich et al. (2010) which is 1.02%. The yield and chemical composition of the leaf oil vary widely between species, individual trees as well as with the growing environment (Robbins, 1983; Penfold and Willis, 1961).

Antifungal activity

The essential oil extracted from the flowers of *E. globulus* was used in the present study to investigate its antifungal potential. *Penicillium citrinum* was used. The results obtained and screening of antifungal activity of essential oil of *E. globulus* are presented in Table 2.

With the agar disc diffusion assay, oils were found to be active against *P. citrinum* at a minimal inhibitory concentration (MIC) of 96.14 µl/ml. The data indicated that *P. citrinum* was the most sensitive strain tested to the oil of *Eucalyptus globulus* with the strongest inhibition zone (11.87mm). Modest activities were observed with minimal inhibitory concentration (MIC) of 3.07 µl/ml. These results are similar to those found by Chang et al. (2008).

The component of this oil, 1.8- cineole, has been known to exhibit antimicrobial activity (Sivropoulou et al., 1997). The antimicrobial activities, in general have been mainly explained through terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils (Belletti et al., 2004). Pinene-type monoterpene hydrocarbons (α-pinene and β-pinene) are well known chemicals having antimicrobial potentials (Dorman and Deans, 2000). The difference in antifungal efficacy is a result of higher concentrations of the same chemical or a result of different chemicals composition between plants.

Several studies have been have been conducted to understand the mechanism of action of plant extracts and essential oils, however it is still unclear. Omidbeygi et al. (2007) suggested that components of the essential oils and extracts cross the cell membrane interact with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and ultimately their death. Cristani et al. (2007) reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but

Table 1. Constituents of the oil of *E. globulus* from Morocco.

Compound	*KI	**Air(%)	Method of identification
A-Thujone	1062	0.10	KI, GC//MS
Humulene	1579	0.31	KI, GC//MS
3-Carene	948	0.10	KI, GC//MS
A-Terpinene	998	0.11	KI, GC//MS
A -Pinene	948	0.20	KI, GC//MS
Camphene	943	0.20	KI, GC//MS
A- Elemene	1410	0.30	KI, GC//MS
A-Cubebene	1344	0.30	KI, GC//MS
Gama-Cadinene	1440	0.30	KI, GC//MS
B-Caryophyllene	1494	0.10	KI, GC//MS
Ocimene	958	0.10	KI, GC//MS
Epizonarene	1469	0.10	KI, GC//MS
Cis-Ocimene	976	0.10	KI, GC//MS
B-Pinene	943	5.20	KI, GC//MS
Isocaryophillene	1494	0.10	KI, GC//MS
Isoledene	1419	0.10	KI, GC//MS
Seychellene	1275	0.16	KI, GC//MS
Copaene	1221	0.20	KI, GC//MS
Ylangene	1221	0.10	KI, GC//MS
Patchoulene	1432	0.10	KI, GC//MS
Sabinene	983	1.49	KI, GC//MS
Isosativene	1339	1.85	KI, GC//MS
Aristolene	1403	2.35	KI, GC/MS
Solanone	1296	6.05	KI, GC/MS
B-Phellandrene	964	0.18	KI, GC/MS
Myrcene	940	1.15	KI, GC/MS
Terpene Hydrochlorite	1116	0.21	KI, GC/MS
Cymene	1042	0.25	KI, GC/MS
Terpenyl Formate	1330	0.20	KI, GC/MS
1,8-Cineole	1059	22.35	KI, GC/MS
Limonene	1018	7.01	KI, GC/MS
Bornyl acetate	1277	0.05	KI, GC/MS
Terpinyl acetate	1333	2.10	KI, GC/MS
Neryl acetate	1352	0.10	KI, GC/MS
A -Eudesmol	1598	0.10	KI, GC/MS
Terpinolene	1052	0.11	KI, GC/MS
Trans-verbenol	1136	4.02	KI, GC/MS
4- Caranol	1125	0.19	KI, GC/MS
Terpinen-4-Ol	1137	3.10	KI, GC/MS
A-Terpineol	1174	1.10	KI, GC/MS
P-Meth-1-En-4-Ol cis	1201	0.15	KI, GC/MS
1-Octen-3-Ol	969	0.17	KI, GC/MS
Geranyl acetate	1352	0.18	KI, GC/MS
Linalyl acetate	1272	0.10	KI, GC/MS
Geraniol	1228	0.19	KI, GC/MS
Linalool	1082	0.21	KI, GC/MS
Carvacrol	1262	0.04	KI, GC/MS
Panasone	2942	0.09	KI, GC/MS
Piperitone	1158	0.14	KI, GC/MS
mentha, 4-8 diene	990	0.19	KI, GC/MS
borneol	1138	0.05	KI, GC/MS

Table 1. Contd.

cis-linalool oxide	1164	0.13	KI, GC/MS
terpinyl isovalerate	1567	0.08	KI, GC/MS
Total Identified Compounds (%)		63.96	
Yields (%)		1.21	

*KI: Kovats Index was determined by GC-FID on a TR5- CPSIL- 5CB column. **Air was determined by mass spectrometry (PlarisQ).

Table 2. Antifungal activity of leaves essential oils of *E. globulus* from Morocco.

Micro-organism	*MIC (µl /ml)	**Disc diffusion assay (inhibition zone mm)
Penicillium citrinum	3.07	4.87
	8.21	5.15
	15.02	5.45
	31.34	6.03
	43.56	9.80
	62.01	9.96
	75.87	11.03
	96.14	11.87

*MIC: Minimal inhibitory concentration, concentration range: 3.07 to 96.14 µl/ml; **Disc diameter 6 mm average of two consecutive trials

also other functions of cell membranes; these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites.

Conclusion

This study revealed a high level of chemical composition of the essential oils of *E. globulus* originated from localities in Atlas median from Morocco. The leaf oil obtained from *E. globulus* was characterized by GC-MS, GC-FID and 54 volatile compounds were identified which made up 63.96% of the total essential oil. The essential oil yields of the studies were 1.21%. The main constituents were 1.8-cineole (22.35%), limonene, (7.01%), solanol (6.05%), β-pinene (5.20%), trans-verbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinene (1.49%), α-myrcene (1.15%) and α-terpineol (1.10%). The fungal strains *P. citrinum* tested were found to be sensitive to essential oils studied and showed a very effective fungicidal activity with minimum inhibitory concentrations (MIC) ranging from 3.07 to 96.14 µl/ml respectively. These results showed that extracts could be considered suitable alternatives to chemical additives for the control of fungal diseases in plants.

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Full Length Research Paper

Evaluation of antibacterial effect of monolaurin on *Staphylococcus aureus* isolated from bovine mastitis

Khayatnouri MH[1]* and Topchi A[2]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary, Tabriz Branch, Islamic Azad University, Tabriz, Iran.
[2]Faculty of Veterinary, Tabriz Branch, Islamic Azad University, Tabriz, Iran.

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Bovine mastitis is the inflammation of the parenchyma of the mammary glands of cattle associated with microbial infections. The most common bacteria causing bovine mastitis include; *Staphylococcus aureus*. Monolaurin, a food grade glycerol monoester of lauric acid, has been reported to have the greatest antimicrobial activity of all of the monoglycerides. The aim of this study was to evaluate of antibacterial effect of monolaurin on *S. aureus* isolated from bovine mastitis in Iran. Milk samples were aseptically collected from each quarter of 20 cows and after isolation and identification of *S. aureus*, 0.5 Macfarland of this bacteria were collected and added to BHI agar with different concentrations of moneulaurin (0, 5, 10, 100, 500, 1000 and 2000 ppm), then *S. aureus* colony count was done and compared to the control (0 ppm of monolaurin). By attention to results of this study monolaurin showed a concentration dependent antibacterial effect. Finally, results of this study demonstrated monolaurin has antimicrobial activity against *S. aureus* isolated from bovine mastitis.

Key words: Bovine mastitis, *Staphylococcus aureus*, monolaurin, colony count.

INTRODUCTION

The nutritional components that make milk an important part of the human diet also support the growth of pathogenic microorganisms coming from milk contamination or from animal infections (Santos et al., 2009). Bovine mastitis is the inflammation of the parenchyma of the mammary glands of cattle associated with microbial infections and physiological changes. Mastitis is caused by a group of infective and potentially pathogenic bacteria, viruses, mycoplasma, fungi and algae. The most common bacteria causing bovine mastitis include; *Staphylococcus* species. In order to minimize the economic losses from bovine mastitis and dissipation of infection resulting from the consumption of contaminated milk and milk products, there is an urgent need to ascertain the current status and involvement of aerobic bacteria in bovine udder inflammation, their role as causative agents of bovine mastitis and their susceptibilities (Dua and Prasad, 2010). *Staphylococcus aureus* is one of the most common etiological agents of bovine mastitis and contributes to significant economic losses in the dairy industry worldwide. While mastitis management programs have contributed to reduced incidence of intramammary infections in recent years, S. *aureus* still persists as a common cause of this disease. In a study in the United States, S. *aureus* and other staphylococcal species were identified in 20.4% of all cows sampled and constituted over 42% of all etiological agents isolated (Wilson et al., 1997). The same organisms were present in 26.5% of cows sampled in a Finnish study, representing 70% of cultures isolated (Honkanen-Buzalski et al., 1996). Staphylococci can be easily transmitted to quarters of lactating cows not only from teat cups and milkers hands during milking but also from the environment via contaminated housing or

*Corresponding author. E-mail: khayat.nouri@gmail.com, mh.khayatnouri@iaut.ac.ir.

pastures. In addition, S. *aureus* is one of the principal causes of clinical mastitis in prepartum or early postpartum heifers (Fox et al., 1995). Non lactating cows are particularly susceptible to staphylococcal infections, and antibiotics are currently routinely administered at drying-off to help eliminate subclinical cases and to prevent new intramammary infections from becoming established. However, the use of antibiotics for prophylactic treatment is subject to considerable debate because of its perceived connection with the emergence of antibiotic resistance in bacteria, particularly with the increased prevalence of organisms such as methicillin-resistant S. *aureus*, which are prevalent in nosocomial infections in humans (Emori and Gaynes, 1993). Such concerns have prompted the World Health Organization to issue recommendations on global programs to try to reduce the use of antibiotics and it is plausible that antibiotic therapies for animals will be restricted in the future, especially as prophylactic agents. Such a limitation would require alternative therapies to be considered, particularly for the control of mastitis in nonlactating cows (Twomey et al., 2000). The use of antibiotics is one of the most commonly used therapies to reduce the intramammary infections caused by pathogens in herds, and the most common reason for treatment was mastitis therapy. Several antibiotics are employed in food animals, among others, β-lactams, tetracyclines, aminoglycosides, macrolides, and sulfonamides (Hoeben et al., 1998). However, the uncontrolled use of these agents has led to appearance of microbial strains more resistant to classic antimicrobials, along with residual antibiotics in milk that are hazardous to public health and interfere with production of dairy products (Souza et al., 2005; Pinto et al., 2001).

Another difficulty in mastitis treatment is the increasing resistance of pathogens to most common antibiotics what limits treatment options (Rossi et al., 2011). There is need to find other antimicrobials that may offer broader spectrum control measures against a wider range of problematic organisms. Lauric acid and other medium chain fatty acids have shown activity against Gram-positive bacteria and yeasts. A monoglycerol ester of lauric acid named Lauricidin®, has been shown to have activity against pathogenic bacteria (Božic et al., 2011). Monolaurin, a food grade glycerol monoester of lauric acid has been reported to have the greatest antimicrobial activity of all of the monoglycerides (Dufour et al., 2007). This study aimed to evaluate of antibacterial effect of monolaurin on *S. aureus* isolated from bovine mastitis in Iran.

MATERIALS AND METHODS

Milk samples were aseptically collected from each quarter of 20 cows and these milk samples (20 mL) were collected in sterilized flasks and stored at 4°C before the analyses. To identify and isolate the micro-organisms, 0.1 mL of each milk sample were spread onto 8% lamb defibrinated blood agar plates and then incubated overnight at 37°C. The microbial strains were presumptively identified on the basis of morphology, hemolysis pattern and Gram staining of the colonies, and then colonies of each microbial strain were streaked on blood agar to obtain a pure culture. Gram positive cocci were tested for catalase and coagulase production according to the standard methods. After identification, colonies of *S. aureus* were stored at -20°C in Brain Heart Infusion enriched with glycerol (1:1 v/v). After this stage 0.5 Macfarland of this bacteria were collected and added to BHI agar with different concentrations of moneulaurin (0, 5, 10, 100, 500, 1000, 2000 ppm) then these plates incubated at 37°C for 24 h, then S. *aureus* colony count was done and compared to the control (0 ppm of monolaurin). Data are presented as mean ± SEM and analyzed statistically using student test and one-way ANOVA followed by Turkey's post hoc test. The level for statistical significance was set at a $P < 0.05$.

RESULTS AND DISCUSSION

Considering the results of this study, monolaurin showed a concentration dependent antibacterial effect. In agreement with the results of the present study, it has been reported by other investigators that monolaurin has antibacterial effects. Monolaurin, like any fatty acid ester, is a lipophilic compound and hence its inhibitory activity is probably through interactions with the cytoplasmic membrane. Although the mechanism of antibacterial action of fatty acids and their derivatives is not defined, it has been suggested to involve disruption of the cell membrane permeability barrier and inhibition of amino acid uptake (Shibasaki and Kato, 1978). The activity of monolaurin against gram-negative bacteria has been shown to be enhanced when combined with high temperatures (Kato and Shibasaki, 1975), freezing (Takano et al., 1979), acidulants (Robach et al., 1981; Smith and Palumbo, 1980), and chelating agents such as EDTA treatments (Branen and Davidson, 2004) which is believed to have increase the ability of the monoglyceride to access the cytoplasmic membrane (Shibasaki and Kato, 1978). Glycerol monolaurate has been shown to inhibit the production of exoenzymes and virulence factors in S. *aureus* (Projan et al., 1994), to block the induction of vancomycin resistance in *Enterococcus faecalis* (Ruzin and Novick, 1998), and to modulate T-cell proliferation (Witcher et al., 1996), all of which involve membrane-bound signal transduction systems. Dodecylglycerol (corresponding ether of monolaurin) has been shown to activate the proteolytic enzyme responsible for the activation of autolysin in the cell wall of *E. faecium* (Ved et al., 1984) and to inhibit glycerolipid and lipoteichoic acid biosynthesis in *Streptococcus mutans* (Brissette et al., 1986). Other studies showed that Lauric acid and 2-nitro-1-propanol suppressed growth slightly during the 24 h of the test. Lauricidin® was the most successful compound tested in suppressing growth of S. *aureus* to less than 1 log at the 24 h mark. Fatty acids have been recognized historically as having antimicrobial activity against Gram-positive bacteria. The mechanism of action of medium and long chain fatty acids against Gram-positive bacteria is thought to be disruption

Table 1. The results of this study in following table have been shown.

Monolaurin (ppm)	0 (Control)	5	10	100	500	1000	2000
Colony count	6566.66 ± 405.51	4900 ± 461.88	4366.66 ± 864.74*	1466.66 ± 145.29*	0 ± 0*	0 ± 0*	0 ± 0*

* P < 0.05 compared with control group.

of cell membranes. Fatty acid toxicity is apparently proportional to the degree of unsaturation. A glycerol monoester of lauric acid, Lauricidin®, has similar activity against gram-postive bacteria to that of lauric acid. In other study showed that Lauricidin® greatly increased the lag phase of S. *aureus* growth, and also kept the growth suppressed for 24 h (Božic et al., 2011).

In another study, it was shown that Lauricidin® was more effective against the Gram positive pathogen L. monocytogenes than against Gram-negatives such as Salmonella Enteritidis or E. coli O157:H7. Also showed that postmilking teat germicides containing Lauricidin® (1%), lactic acid (6%), and lauric acid (0.85%) against S. *aureus*, and reported numbers reduced by nearly 80% (Božic et al., 2011). Preuss et al. (2005) examined the effects of a variety of essential oils and monolaurin on two *S. aureus* strains. In that study, origanum oil was the most potent of the essential oils tested and, in cultures of the two S. *aureus* strains, proved bactericidal at 0.250 mg/mL. *In vitro*, monolaurin's effects mirrored those of origanum oil. The combination of monolaurin and origanum oil was bactericidal at the 0.125-mg/mL concentration of each. In two separate experiments *in vivo*, intraperitoneal injections of S. *aureus* killed all 14 non-treated mice within the period of 1 week. In treated mice, more than 40% (6 of 14) survived for 30 days when given oral origanum oil daily. Fifty percent of the mice survived for 30 days when receiving daily vancomycin (7/14) and monolaurin (4/8). More than 60% of mice survived when receiving a daily combination of origanum oil and monolaurin (5/8) (Preuss et al. 2005). In the study of Preuss et al. (2005), they assessed in vitro the static and/or cidal activity of various essential oils (particularly wild Mediterranean oregano) and the monoglycerides of lauric acid (monolaurin) against gram-positive, gram-negative, and acid-fast microorganisms. They examined *Bacillus anthracis* Sterne, grown from a live veterinary vaccine and Mycobacterium terrae as surrogates for virulent *B. anthracis* and *Mycobacterium tuberculosis,* respectively. In addition, they examined S. *aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Helicobacter pylori* (Preuss et al., 2005). Monolaurin is another natural substance composed of monoglycerides and fatty acids with potential antimicrobial properties. In other study champions the use of certain lipids as antimicrobials. They measured the optimum antimicrobial activity for fatty acids and their corresponding monoglycerides and reported that the optimum chain length for therapy is 12 carbons (C12). Lauric acid (C12) has greater antiviral activity than caprylic acid (C8), capric acid (C10), or myristic acid (C14). In contrast to monolaurin, the dilaurin derivative was inactive. It is now generally accepted that monoglycerides are active whereas diglycerides and triglycerides are inactive against microorganisms (Kabara, 1980). In one study of several fatty acid esters of polyhydric alcohols, a broth-dilution methodwas used to determine the MICs effectiveness against gram-negative and gram-positive organisms. Of the tested compounds, gram-positive organisms were affected to the greatest extent by monolaurin (Conley and Kabara, 1973). In general, gram-negative organisms were not affected. Monolaurin is effective in blocking or delaying production of exotoxins by pathogenic gram-positive bacteria (Schlievert et al., 1992) and inhibits the synthesis of most staphylococcal and other exoproteins at the level of transcription (Projan et al., 1994). Monolaurin also inhibits signal transduction pathways and, thereby, the expression of virulence factors including protein A, alpha-hemolysin, Blactamase, and toxic shock syndrome toxin 1 in *S. aureus* and the induction of vancomycin resistance in *E. faecalis* (Ruzin and Novick, 2000). When the bactericidal effects of several fatty acids and monoglycerides on Chlamydia trachomatis bacteria were studied *in vitro*, the cidal effects appeared to be related to the disruption of the membrane of the elementary body (Bergsson et al., 1998). Corroborating that finding are those of viral studies that suggest the inactivation effects are due to membrane disintegration caused by fatty acids (Isaacs and Thormar, 1991), a finding similar to that in a report of the action of origanum oil on viruses (Siddiqui et al., 1996). Concerning gram-negative organisms, the failure of monolaurin against *E. coli* and *K. pneumoniae* was expected, because monolaurin is known to kill primarily gram-positive organisms (Enig, 1998). However, this study corroborates previous findings (Petschow et al., 1996) showing that monolaurin is exquisitely effective against *H. pylori*, a gram-negative organism that is difficult to culture. Because

approximately two-thirds of the world's human population is colonized or infected with this organism, a safe and effective herb-derived are in agreement with others who showed that monolaurin produce antimicrobial activity against pathogen bacterial.

Conclusion

Results of this study demonstrated that monolaurin has antimicrobial activity against *S. aureus* isolated from bovine mastitis.

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Full Length Research Paper

Efficacy of losartan and combination of losartan plus hydrochlorothiazide for high blood pressure treatment

Bunc Matjaž[1, 2, 3] and Šušteršič Miha[1]

[1]Department of Cardiology, University Medical Centre Ljubljana, Zaloska c. 007, 1000 Ljubljana, Slovenia, Europe.
[2]Institute for Pathophysiology, Medical Faculty, University of Ljubljana, Zaloska c. 002, 1000 Ljubljana, Slovenia, Europe.
[3]Center for Medical Therapy and Research, Cerklje, Slovenia, Europe.

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Hypertension is one of the most common cardiovascular risk factors in adult population. Even values of high normal blood pressure are linked to increased risk of major cardiovascular events and only minority of patients reach the target values with antihypertensive medications prescribed. We wanted to demonstrate the efficacy and safety of treatment with losartan and/or combination of losartan plus hydrochlorothiazide in middle aged population with most frequent cardiovascular risk factors. We conducted a multicenter, prospective, observational, non-randomized study over 10 weeks in patients with essential hypertension whose blood pressure was not adequately controlled despite previously prescribed antihypertensive therapy. Main outcome parameters were systolic and diastolic blood pressure reduction and the rate of normalization of blood pressure at study end, comparing to baseline. 595 patients were included in the study, 313 men and 281 women (1 of the participant's sex was not determined), with mean age of 63.1 ± 10.0 years. Patients received losartan and/or losartan plus hydrochlorothiazide instead or on top of their previous antihypertensive medications. Average blood pressure was lowered from (158 ± 13)/(95 ± 9) mmHg to (137 ± 10)/(84 ± 8) mmHg and (157 ± 15)/(92 ± 9) mmHg to (136 ± 12)/(82 ± 8) mmHg in non-diabetic and diabetic patients respectively (*P*<0.05). Average blood pressure reduction was 21/11 mmHg for non-diabetic patients while reduction of blood pressure for diabetic patients was 20/11 mmHg. Target blood pressure attainment rates were higher in non-diabetic patients. From our results we may conclude that losartan and/or combination of losartan plus hydrochlorothiazide are effective and potent antihypertensive agents and could be used in high risk non-diabetic and diabetic patient due to its neutral and some beneficial metabolic effects.

Key words: Losartan, hydrochlorothiazide, high blood pressure.

INTRODUCTION

Arterial hypertension is one of the most common cardiovascular risk factors in adult population [Summary of the 2007 European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines, 2007; Stergiou et al., 2004; Kearney et al., 2005; Kannel, 1996], especially for myocardial infarction and stroke (Bronner et al., 1995; Kjeldsen et al., 2001). Arterial hypertension leads to left ventricular hypertrophy (LVH) which strongly predicts myocardial infarction, stroke, and cardiovascular death (Koren et al., 2001; Mensah et al., 1993) in the general population (Levy et al., 1990; Bikkina et al., 1994) and patients with coronary artery disease (Ghali et al., 1992). The duration of hypertension and values of blood pressure influence the risk of stroke, heart failure, atherosclerosis and kidney disease (Kannel, 1996). The Framingham Heart Study showed that people with blood pressure (BP) values of 130 to 139/85 to 89 mmHg have more than two times the risk for developing cardiovascular diseases, than patients with BP ≤ 120/80 mmHg (Vasan et al., 2001).

*Corresponding author. E-mail: mbuncek@yahoo.com. Tel: +386-1-522-28-44. Fax: +386-1-522-28-28

The prevalence of arterial hypertension is immense, especially in Europe. Age- and sex-adjusted prevalence of hypertension was 28% in the North American countries and 44% in the European countries at the 140/90 mmHg threshold (Wolf-Maier et al., 2003). Although hypertensive patients are readily recognized, only minority of them reach blood pressure goals with treatment, especially in Europe. Compliance to pharmacological therapies plays an important role in achieving blood pressure goals in hypertensive patients (Wolf-Maier et al., 2004). Inadequate compliance of some patients may be due to unpleasant side effects of prescribed drugs (Bangalore et al., 2007) or too many different medications prescribed.

The European Guidelines recommend prompt treatment of patients affected by hypertension to reduce cardiovascular risk [Summary of the 2007 European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines, 2007]. Angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), diuretics, beta-blockers, calcium channel-blockers, alpha adrenergic blockers and seldom other antihypertensive agents may be used to treat arterial hypertension [Summary of the 2007 European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines, 2007; Stergiou et al., 2004]. When arterial hypertension is diagnosed, the target blood pressure values are rarely achieved by only one antihypertensive medication. Because of that, there are several fixed dose-combinations now available on the market.

Since approval of first ARB – losartan (L) for clinical use, several other ARBs emerged as potent antihypertensive medications (valsartan, candesartan, irbesartan, telmisartan, and olmesartan) (Oparil et al., 2001; Wen-peng et al., 2011). L is an ARB, whose efficacy and potency was clearly established in LIFE study, which showed significant reduction, not only of the blood pressure, but also of the cardiovascular morbidity and mortality comparing to atenolol (Lindholm et al., 2002; Dahlöf et al., 2002). Hydrochlorothiazide (H), a benzothiazide diuretic, inhibits tubular sodium and chloride reabsorbtion and with that promotes natriuresis. Although its exact mechanism of action is unknown, H has complementary effect to ARB that makes them a good combination as antihypertensive regiments (Kjeldsen et al., 2005; Brunner et al., 1980).

The aim of this study was to demonstrate the efficacy of a prompt pharmacologic treatment with L or combination of losartan plus hydroclorothiazide (LH) as a mono-therapy or add-on therapy in middle aged population with most frequent cardiovascular risk factors.

MATERIALS AND METHODS

In open-label, multicenter, prospective, observational, non-interventional, non-randomized and post-marketing surveillance study, we evaluated effectiveness of treatment with L alone (maximum daily dose of 100 mg) and the fixed combination of LH (maximum daily dose of 100 mg/25 mg) in reaching blood pressure goals. L or LH served either as mono-therapy, exchange for an existing therapy or add on therapy. Study was approved by the Slovenian ethic committee in research and was observational and neither randomized, nor blind. Every patient served as a control to himself. Study had begun in September 2008 and ended in November 2009. The follow up of individual participant in the study was 8 to12 weeks (median of 10 weeks). All the participants were recruited in Slovenia. 75 physicians (specialists of internal medicine, primary care physicians) recruited up to 10 consecutive patients for the study. Only adult patients (18 years or older) were included after they signed their informed consent about participation in the study. The decision on which of the studied medication was prescribed to an individual patient and the way in which physicians controlled the course of treatment and which concurrent medication they prescribed, was not influenced. All the adverse events were obliged to be reported to the manufacturer, who then forwarded the report in a standardized form to the relevant authorities. Two criteria (inclusion and exclusion criteria) were used in this study. Inclusion criteria entail adult patients who: (1) have known essential hypertension, or (2) are already treated for arterial hypertension comprising (a) arterial blood pressure ≥ 140/90 mmHg, (b) diabetes and arterial blood pressure ≥ 130/80 mmHg, or (c) high or very high risk regarding their risk stratification and arterial blood pressure ≥ 130/80 mmHg; whereas exclusion criteria entail: (1) adequately controlled blood pressure regarding their risk stratification, (2) known side effects on L or LH, or (3) known hypersensitivity to L or LH.

At first visit and inclusion in the study we assessed the risk factors for cardiovascular disease (age, arterial blood pressure, dyslipidemia, body mass index (BMI), waist circumference and smoking) (Table 1). Blood pressure was taken with available manometer with upper-arm cuff in the individual physicians practice after five minutes in a sitting position. The average of three blood pressure measures was accounted, which was always measured on the same individual upper-arm. From blood samples, which were taken at inclusion in the study, values of total cholesterol (CH), low density lipoproteins (LDL), high density lipoproteins (HDL), fasting blood glucose level, glycosylated hemoglobin (HbA1C), serum potassium, serum creatinine were determined. Signs of target organ damage were accounted if they were already stated in the patients chart (their last measurement). At the end of the study arterial blood pressure, BMI and waist circumference were measured and blood was taken to asses metabolic effect of treatment (CH, LDL, HDL, fasting blood glucose, HbA1c, serum potassium and serum creatinine).

We used paired Students t-test to calculate the statistical significance of the differences between all the measured variables between the beginning and the end of the study. All data was expressed as means ± SD. Statistical significance was set at $P<0.05$. The analysis was performed with the statistical software SPSS 17.0 for Windows.

RESULTS

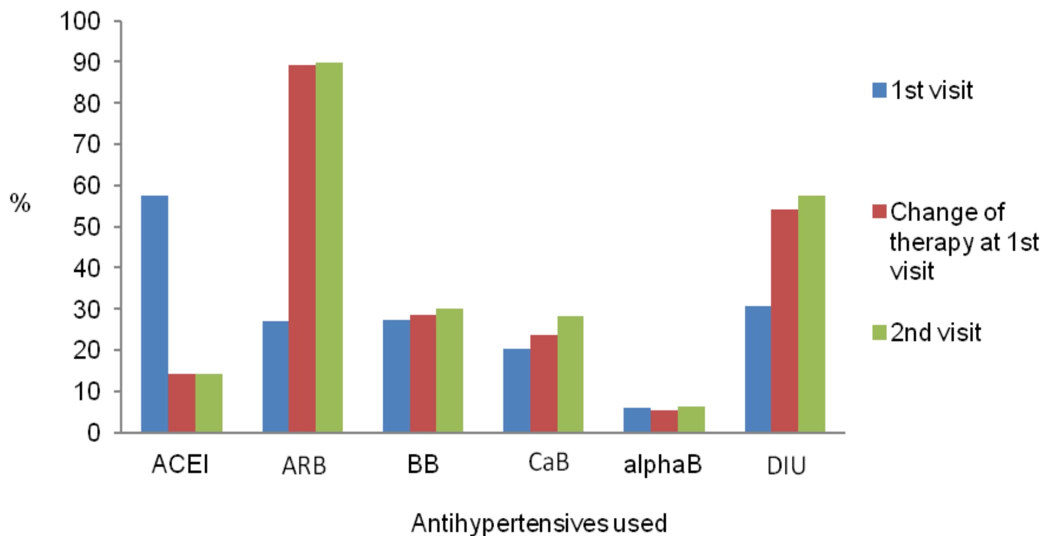
Baseline characteristics

The total number of patients included was 595; there were 313 men and 281 women (1 of the participant's sex was not determined). 15 patients did not finish the study or the protocol was not filled correctly. In total cohort, men were more frequent than women. The mean age of the patient in the study was 63.1 years (±10 years), ranging from 24 to 87 years (Table 1).

Among those who had an echocardiography and Doppler ultrasound of carotid arteries done before the

Table 1. Baseline characteristics. BMI – Body mass index.

	Men	Women
Average age (years)	60.9	65.5
Average BMI (kg/m²)	29.6	29.3
Average Waist circumference (cm)	101.8	94.3
Diabetes (%)	51.9	46.6
No diabetes (%)	48.1	53.4
Smoker (%)	25.5	24.7
Dyslipidemia (%)	67.2	61.8
Early cardiovascular death in family (%)	36.1	39.9
Abdominal obesity (%)	57.7	57.5
Metabolic syndrome (%)	55.8	59.1



Graph 1. Antihypertensive therapy in the study. Antihypertensive therapy used before inclusion in the study, changes to antihypertensive therapy at 1st visit and antihypertensive therapy at 2nd visit of all participants in the study expressed in %; ACEI – angiotensin converting enzyme inhibitor, ARB – angiotensin receptor blocker, BB – beta blocker, CaB - calcium channel blocker, alphaB – alpha blocker, DIU – diuretic.

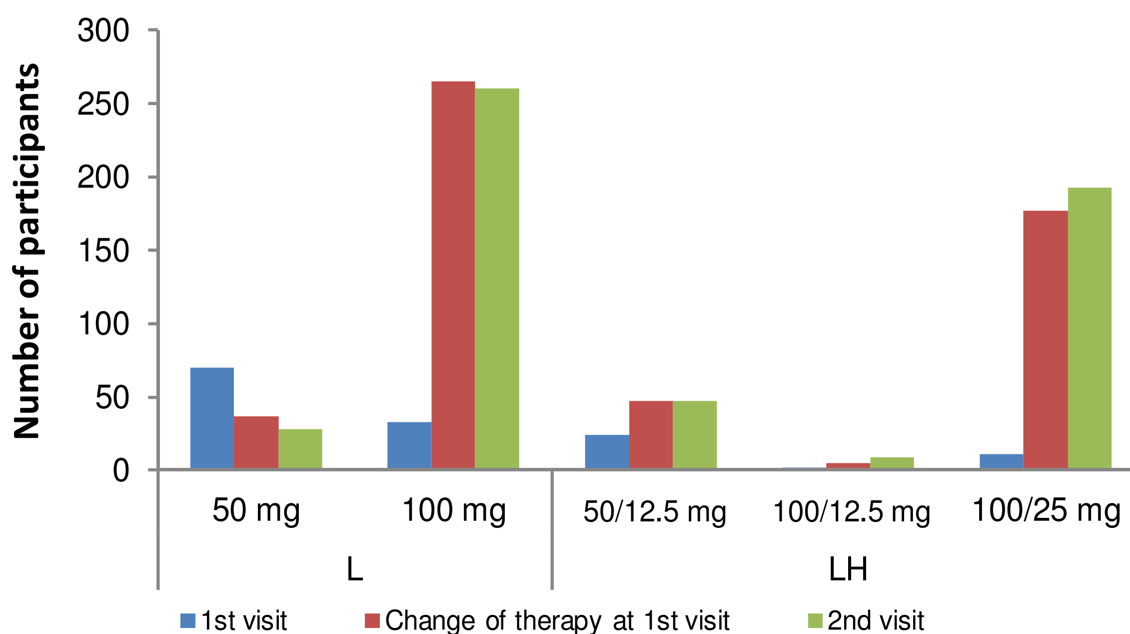
study, 35.8% had signs of left ventricular hypertrophy and 15.5% had atherosclerotic plaques. Besides hypertension and diabetes there were also other co-morbidities that influence participant's long term prognosis: Heart disease (26.8%), peripheral vascular disease (14.3%), cerebro-vascular disease (9.9%), renal disease (8.6%) and retinopathy (7.3%) (Table 2). The patients' compliance to the treatment was not assessed.

Antihypertensive pretreatment and treatment

Patients were included in the study mostly due to an inadequate blood pressure control on their medical treatment or ACEI intolerance. Many different antihypertensives were used: ACEI, ARB, calcium channel blockers, diuretics, beta blockers and alpha blockers (Graph 1). 94.4% of all patients were treated with antihypertensive medications prior to study entry. At the beginning of the study 36.2% had only one antihypertensive agent, 38.7% had two, 16% had three and 2% had 4 antihypertensive agents. The majority of included patients had some co-morbidities and therefore concomitant medications. At inclusion, 66.9% of participants did not have any adverse effects of their treatment before inclusion in the study. The most common

Table 2. Estimated risk for a major cardiovascular event in 10 years at baseline.

Estimated risk for major cardiovascular event in 10 years (%)	Men (%)	Women (%)	Total (%)
< 15	10.1	13.8	12.4
15 – 20	27.6	22.9	25.4
20 – 30	31.7	35.8	33.6
> 30	30.6	27.6	28.5

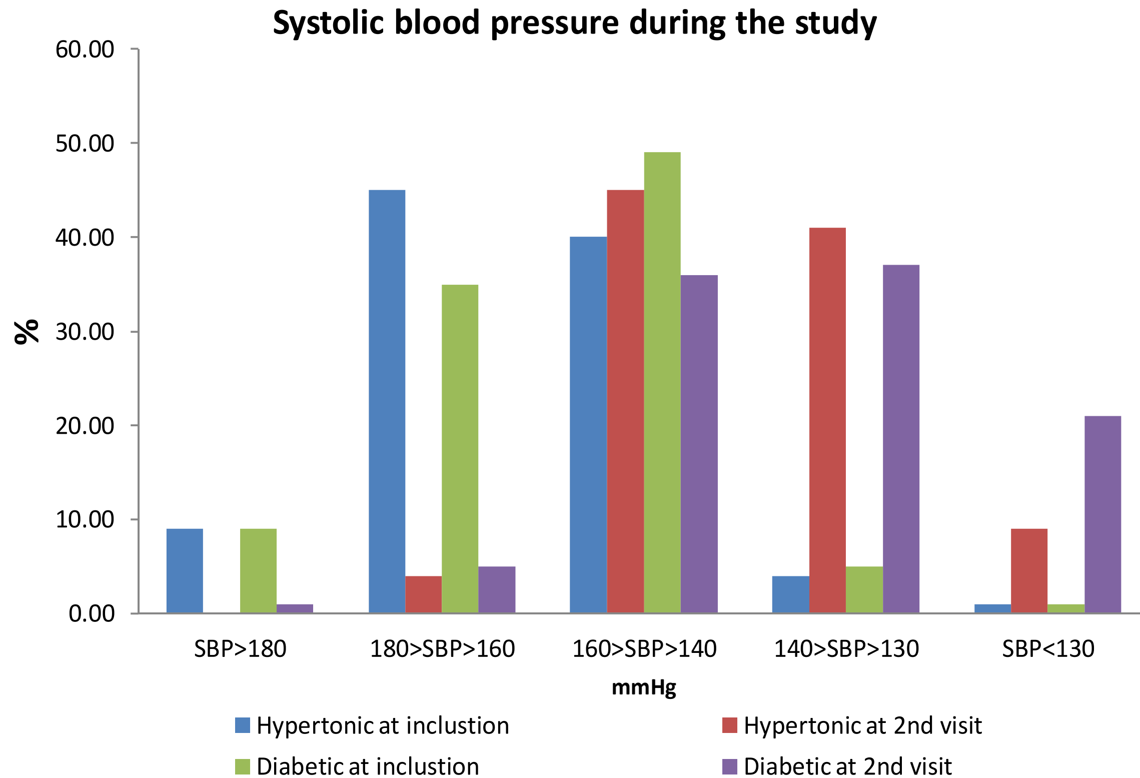


Graph 2. Usage of L or LH in different dosages. Usage of L or LH regarding their dose before inclusion in the study, changes to antihypertensive therapy at 1st visit and antihypertensive therapy at 2nd visit of all participants in the study. L – losartan, LH – losartan + hydrochlorothiazide;

adverse effect of their antihypertensive treatment was dry cough in 24.4% (if they were treated with ACEI) while all other side effects were present in 8.7%. L was introduced most frequently in 100 mg (47%) and LH in 100/25 mg (31%). At the end of the study the most frequently used doses of L were 100 mg (46%) and LH 100/25 mg (34%) (Graph 2).

Blood pressure

Average blood pressure at the beginning of the study of all non-diabetic participants was (158 ± 13)/(95 ± 9) mmHg and (157 ± 15)/(92 ± 9) mmHg for diabetic patients, respectively. At the end of the study, the average blood pressure was (137 ± 10)/(84 ± 8) mmHg for non-diabetic patients and (136 ± 12)/82 ± 8) mmHg for diabetic patients. At the beginning of the study, 51% of non-diabetic patients had systolic blood pressure (SBP) over 160 mmHg and 45% had diastolic blood pressure (DBP) over 100 mmHg. Only 5% of the non-diabetic patients had SBP below 140 mmHg and 6% had DBP below 90 mmHg. At the end of the study 50% of the non-diabetic patients had SBP below 140 mmHg and 58% had DBP below 90 mmHg. At the beginning of the study 44% of diabetic patients had SBP over 160 mmHg and 28% had DBP over 100 mmHg. Only 1% of the diabetic patients had SBP below 130 mmHg and 17% had DBP below 80 mmHg. At the end of the study 21% of diabetic patients had SBP under 130 mmHg and 66% diastolic



Graph 3. Systolic blood pressure (SBP) of hypertonic and diabetic patients during the study expressed in %.

blood pressure under 80 mmHg (Graph 3, Graph 4).

Laboratory values

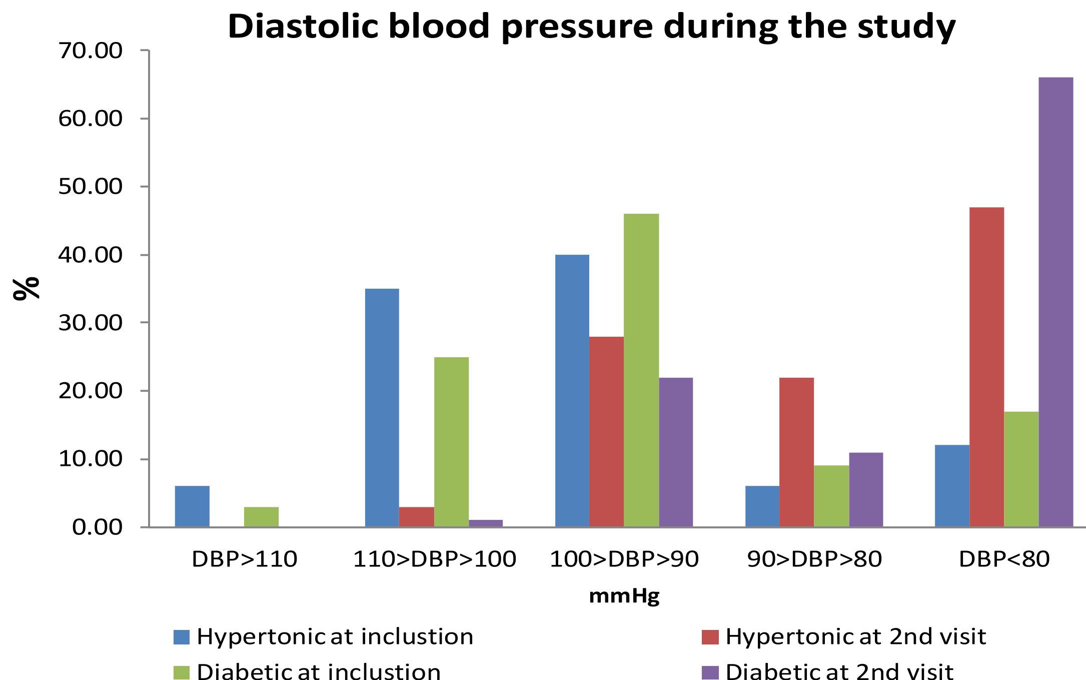
Treating non-diabetic patients with L and/or LH on top of or instead of their former therapy made no significant differences on waist circumference, CH, HDL, fasting blood glucose, serum potassium and creatinine ($P>0.05$). There was a statistical significance in improvement of LDL levels and BMI after treatment with L and/or LH ($P<0.05$). Average LDL levels were reduced from 3.3 to 3.0 mmol/L, while average BMI levels were reduced from 28.4 to 27.7 kg/m^2. Treating diabetic patients with L and/or LH on top of or instead of their former therapy made no significant differences on BMI, waist circumference, HDL, HbA1c, serum potassium and creatinine levels ($P>0.05$). There was a statistical significance in improvement in CH, LDL and fasting blood glucose levels after treatment with L and/or LH ($P<0.05$). Average CH levels were reduced from 5.8 to 5,3 mmol/L, average LDL levels were reduced from 3.5 to 3.0 mmol/L and average fasting blood glucose levels were reduced from 7.1 to 6.4 mmol/L.

Physician's perspective on efficacy of L and LH

Two main reasons for a change in antihypertensive therapy at the beginning of the study where dry cough and inadequately controlled blood pressure. 85% of the physicians were satisfied with L and/or LH treatment at the end of the study, because blood pressure goals were reached. 15% of the physicians were unsatisfied because of different causes, the most frequent of them were the inadequately controlled arterial blood pressure (7%) and other causes – not specified (8%).

DISCUSSION

This study examined the antihypertensive efficacy of 10 week treatment with L and/or LH as mono therapy or as add on antihypertensive therapy in middle aged population with most frequent cardiovascular risk factors. Since hypertension is a multi-factorial disease, combining therapies with different mechanisms of action, can additively reduce blood pressure. The benefit of adding L to H may be explained by the fact that diuretics decrease the intravascular volume activating renin-angiotensin-aldosteron system resulting in a diminished antihypertensive response, on which L works and blocks it. Regarding that L in combination with H has additional BP lowering effects compared to the mono therapy (Kjeldse et al., 2005; Brunner et al., 1980). The endpoint study available, serving as the basis of approval of the high-dose L or LH combination, was the randomized



Graph 4. Diastolic blood pressure (DBP) of hypertonic and diabetic patients during the study expressed in %.

controlled Losartan Intervention For Endpoint reduction in hypertension (LIFE) study (Lindholm et al., 2002; Dahlöf et al., 2002).

With the exception of two German (Bönner et al., 2009; Förster et al., 2007), one Spanish (Coca et al., 2002) and one Japanese (Saruta et al., 2007) study, to our knowledge, there are no current data on the efficacy and safety of the unselected “every-day” care patients in primary care settings. However, high-dose of L and LH is less documented than lower doses of L and LH.

Efficacy

The results of the study showed that the treatment with L or LH in middle aged population with most frequent cardiovascular risk factors is very effective, which was also shown before (Kjeldse et al., 2005; Brunner et al., 1980). The average reduction of blood pressure for non-diabetic patients was 21/11 mmHg and 20/11 mmHg for diabetic patients, respectively. The mean reduction of SBP and DBP was comparable to other studies already done. Fixed-dose combination improves medication compliance for 24 to 26% (Bangalore et al., 2007). These data are in agreement with the well known tolerability of other ARB´s including losartan as mono-therapy or combination therapy.

Target level achievement

It is well known that arterial hypertension is readily recognized in European countries, but blood pressure goals are reached only in minority patients treated (Wolf-Maier et al., 2003; Wolf-Maier et al., 2004). It seems that the same is true for Slovenia. Regarding that majority of the patients included in the study were already known and treated for arterial hypertension (only 5% of patients included in the study did not have prior antihypertensive therapy), SBP goals (SBP below 140 mmHg) at inclusion in the study were achieved in only 5% and DBP goals (DBP under 90 mmHg) in only 18% of non-diabetic patients (Graph 3). Of those who have, besides hypertension also diabetes, only 1% had SBP and only 17% had DBP within the target limits at inclusion (arterial blood pressure below 130/80 mmHg) (Graph 3, Graph 4). Even though that L and LH substantially lowered blood pressure during the study, SBP goals were reached in 50%, while DBP goals were reached in 69% of those who had hypertension without diabetes. In patients that had high blood pressure and diabetes, DBP goals were reached more easily than SBP goals with L or/and LH treatment. Only 21% of patients that had arterial hypertension and diabetes reached SBP goals and 66% reached DBP goals. The reason for that may be in greater arterial wall stiffness in patients with arterial hypertension and diabetes.

Effects of treatment with L and/or LH on other measured parameters

There were no important changes in serum potassium, creatinine and HDL levels in non-diabetic and diabetic patients (P>0.05). Waist circumference in all the included

patients was also not significantly changed (*P*>0.05), while BMI (the reduction was rather small) was significantly lower at the end of the study in non-diabetic patients (*P*<0.05). It is interesting that fasting serum glucose level was not a subject of significant change in non-diabetic patients but it was significantly lower at the end of the study in diabetic patients (*P*<0.05). Fasting serum glucose could be the subject of a quite substantial change during the day, especially in diabetic patients, while HbA1c represents serum glycemic control on a longer term. HbA1c at the end of the study was not significantly changed in diabetic patients (*P*>0.05), so we may conclude that L and LH are not affecting the metabolism of glucose even though our study showed an improvement in the serum glucose after treatment with L and/or LH in diabetic patients. We also found an improvement in serum LDL levels in all the patients at study end (*P*<0.05), which is not in concordance with studies done before (Moen et al., 2005). It is interesting that our study showed an improvement in total serum cholesterol in diabetic patients (*P*<0.05), while there were no improvement in non-diabetic patients. Although the reduction of total serum cholesterol in diabetic patients and reduction of LDL levels in all included patients reached the level of significance, they were rather small. Other potentially beneficial effects of L and LH were not assessed in this study. However, in studies conducted before it was shown, that L has a capacity to reduce microalbuminuria, because of blocking the renin-angiotensin-aldosteron system largely independent of its pressure lowering effect, which makes this agent particularly suitable for patients with renal disease (Brenner et al., 2001).

Methodological considerations

When interpreting the present study, certain methodological aspects have to be taken into account. Selection bias is to be expected despite physicians being requested to include eligible patients consecutively. The trial was not controlled, so the placebo effect cannot be accounted for. Long term influence of the treatment on mortality, target organs ischemia and heart failure could not be analyzed from our data because the duration of the study was only 12 weeks at most. Laboratory data was measured only twice and only approximately 10 apart. Some of the laboratory values were measured only once, so they could not be compared to their previous value and had to be excluded. We also did not monitor a 24h antihypertensive profile, so we cannot say anything about sustainment of antihypertensive effect of L and LH during the day.

Conclusions

From our results we may conclude that losartan and/or combination of losartan plus hydrochlorothiazide are effective, potent antihypertensive agents and could be used in high risk non-diabetic and diabetic patients due to its neutral and some beneficial metabolic effects.

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Full Length Research Paper

Lycium barbarum polysaccharides (LBP) extraction technology and its antioxidation activity

Ling Yuan[1,2], Yi Nan[3], Rong Wang[3] and Yang Niu[3]*

[1]Pharmacy College of Ningxia Medical University, Yinchuan, Ningxia Province.750004, People's Republic of China.
[2]Minority Traditional Medical Center of Minzu University of China, Beijing.100081, People's Republic of China.
[3]Traditional Chinese Medicine College of Ningxia Medical University, Yinchuan City, Ningxia Province, 750004, People's Republic of China.

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This study was carried out to investigate the technology of extracting *Lycium barbarum* polysaccharide (LBP) and its antioxidation activity, using orthogonal experimental method to optimize its extraction conditions. Fenton reaction and pyrogallol experimental method were adopted to determine the antioxidation activity of LBP. The three factors investigated in the orthogonal experiment affected the results. Different concentrations of LBP effectively cleared hydroxyl radical (OH^-) and superoxide anion (O_2^+). It was concluded that the extraction of LBP by cold maceration as well as the three factors, (solvent volume, extraction time and extraction times) affects its yield, and that it has antioxidation activity.

Key words: *Lycium barbarum* polysaccharides, antioxidation activity.

INTRODUCTION

Barbary wolfberry is a beneficial traditional Chinese medicinal food. Barbary wolfberry, belonging to Solanaceae family, *Lycium*, is a perennial deciduous shrub, has more than 80 species in the whole world and is widely distributed mainly in North and South America and Eurasia. In China, it is mainly distributed in Gansu, Ningxia, Tianjin and other places. Ningxia wolfberry is considered the best (He et al., 1997). Barbary wolfberry contains a variety of active ingredients, including carbohydrate, amino acids, trace elements, vitamins, alkaloids, fatty acids, etc; of it species, *Lycium barbarum* polysaccharide (LBP) has the highest carbohydrate content and its total carbohydrate content is up to 46.5% (Jiangsu, 1977).

In modern pharmacology, it is confirmed that polysaccharides plants clear oxygen radicals, increase antioxidase activity, resist lipid peroxidation, reduce the production of PGs, LTs, malonaldehyde (MDA) and other lipid peroxidation metabolites. They also do not allow cell metabolism and morphological changes caused by molecular aggregation and cross linking of membrane proteins and enzymes; and finally they clear free radicals and delay senility. Some studies (Dai et al., 2010) show that barbary wolfberry can reduce serum MDA content in elderly mouse and increase the biological activity of superoxide dismutase (SOD). This indicates that it can effectively increase antioxidase activity, clear oxygen radicals and reduce lipid peroxidation level. That shows it has good antioxidation activity.

This experiment extracts, separates and purifies LBP, to prove its antioxidation activity and explore its action mechanism.

*Corresponding author. E-mail: nanyiailing10@sohu.com. Tel: +86 0951-4091605.

Table 1. Investigation of the levels of the factors for LBP extraction technology.

Level	Factor 1	Factor 2	Factor 3
	Extraction time (h) A	Extraction times B	Solvent volume (ml/g) C
1	1	1	40
2	2	2	60
3	3	4	80

MATERIALS AND METHODS

Kunming mice with body weight of 20 ± 2 g were bought from the Institute of Laboratory Animal Sciences of CAMS; the needed reagents were domestically obtained and were of analytical grade.

Investigation of LBP extraction technology

Pretreatment

Ningxia wolfberry was dried at 60°C to constant weight, crushed and screened through a 100 mm diameter-mesh. The filtrate was stored for later use.

Orthogonal experimental design for LBP water extraction technology

LBP is a water-soluble polysaccharide, and its content in Barbary wolfberry is above 35%. It os mainly composed of arabinose, galactose, glucose, rhamnose, mannose and xylose (Huang et al., 1998). In order to increase LBP extraction efficiency, this experiment investigates the three main factors affecting the results namely, solvent volume, extraction time and extraction times. The levels of the factors are shown in Table 1. To precisely weigh 9 shares of sample and number them, the experiment was carried out in a 3 × 3 orthogonal design and LBP content was calculated.

Hydroxyl radical (OH^-) clearing effect (Jin et al., 1996)

H_2O_2/Fe^{2+} system (Chun and David, 2001) is adopted to generate OH^- by Fenton reaction. Precisely weighed 1.5 ml phenanthroline solution (5 mmol/L) was added to 1.0 ml phosphate buffer (pH = 7.4), and simultaneously 0.2 ml 7.5 mmol/L $FeSO_4$ and 1.0 ml 0.1% H_2O_2 were added. Immediately, it was mixed. Water was added to total volume of 10 ml thermostatic water bath at 37°C for 1 h; then the absorbance was measured at 536 nm. In the experiment, LBP extracts were prepared at different concentrations (original herbs had 200 mg, 400 and 600 mg/L) with the aforementioned methods, and A_{536} (sample) was measured. A_{536} (undamaged) was measured without adding sample and H_2O_2. The clearance rate was then calculated.

$$d = [A_{536}\text{ (sample)} - A_{536}\text{ (damaged)}] / [A_{536}\text{ (undamaged)} - A_{536}] \times 100\%$$

Superoxide anion (O_2^+) clearing effect (Zhang et al., 2004)

To adopt pyrogallol autoxidation in order to initiate *in vitro* chemical reaction, 4.5 ml 1 mmol/L Tris-HCl buffer (pH 8.2) was put in a test tube and preheated at 25°C for 20 min; 0.2 ml 3 mmol/L pyrogallol and 2 ml LBP solution of different concentrations were added to water bath at 25°C for accurately 4 min. Then A_{325} value, denoted as A_{sample} was measured. The reaction solution without extract was taken as the reference. A_{325} value, denoted as $A_{damaged}$, was measured and the clearance rate was calculated as follows:

$$\text{Clearance rate} = (A_{damaged} - A_{sample}) / A_{damaged} \times 100\%$$

RESULTS

Orthogonal experiment results

The results show that the amount of water, extraction time and extraction times affect LBP extraction yield. The ranges of the factors are 2.97, 1.16 and 5.65, showing that the impacts on the experimental results are C>A>B. The highest LBP content is 18.66% and the lowest is 12.56%, indicating that $A_3B_1C_3$ (that is, 80 ml/g water, once and 3 h extraction) is the best option (Table 2).

Experimental result for LBP clearing hydroxyl radical (OH^-)

The results in Table 3 show that LBP has significant clearing effect on hydroxy radical (OH^-). As the concentration of the test sample increases, the radical clearing ability of LBP increases gradually.

Result of clearing effect of LBP on superoxide anion (O_2^+)

The result shows that LBP of different concentrations has certain clearing effect on superoxide anion O_2^+, and the clearing effect presents dose-effect linear relationship as the dose increases (Table 4).

DISCUSSION

In this investigation of extraction technology, the extraction method involves cold maceration, without heating, and the main consideration is that polysaccharide is changed easily in high-temperature extraction process. This affects its yield and thereby affects the accuracy of

Table 2. Orthogonal experiment results for LBP extraction technology.

Experiment no.	Factor 1	Factor 2	Factor 3	LBP extraction yield (%)
	Extraction time (h) A	Extraction times B	Solvent volume (ml/g) C	
1	1	1	1	12.56
2	1	2	2	14.85
3	1	3	3	15.21
4	2	1	2	16.32
5	2	2	3	17.54
6	2	3	1	15.92
7	3	1	3	18.66
8	3	2	1	14.59
9	3	3	2	17.67
K_1	18.65	19.61	17.94	-
K_2	20.83	20.73	19.58	-
K_3	21.62	20.77	23.58	-
R	2.97	1.16	5.65	-

Table 3. Experimental result for LBP clearing hydroxy radical (OH^-).

Test sample	Concentration (mg/L)	OH^- clearance rate (%)
1	200	18.5
2	400	45.7
3	600	68.3

Table 4. Result of clearing effect of LBP on superoxide anion O_2^+.

Test sample	Concentration (mg/L)	O_2^+ clearance rate (%)
1	200	15.36
2	400	48.69
3	600	62.51

the results. In addition, some pigments in barbary wolfberry dissolve at high temperature and react easily with polysaccharides. This does not only affect the polysaccharide technology results, but can also bring hidden troubles to subsequent polysaccharide antioxidation experiments. Free radicals are groups or molecules with unpaired electrons and generally have short life, are unstable, active in nature etc.. Under normal circumstances, the human body produces appropriate amount of free radicals for maintaining normal operation of the body. However, as age increases, free radical metabolism in tissues and body fluids slows down, excessive free radicals remain in the body, and the damage caused by free radical reactions leads to human aging and diseases.

Currently, synthetic antioxidants can inhibit oxygen radical reactions, but excessive oxygen radicals in the body can cause bio-membrane damage, protein denaturation, enzyme inactivation and deoxyribonucleic acid (DNA) replication error, thus causing various diseases in the body (Valavanidis et al., 2004; Pincemail et al., 2002). Valavanidis et al. (2004); Olorunnisola et al. (2012) and Saalu et al. (2012) found that cancer (Srivastava and Mittal, 2005; Kannan, 2006), cardiovascular and cerebrovascular diseases (Sagar et al., 1992; Olfat et al., 2012), acquired immune deficiency syndrome (AIDS) and other diseases are related with oxygen radicals; therefore, the adverse effects caused by oxidation reactions coupled with oxygen radicals are of high concern and have gradually become research hotspot. OH^- is active oxygen with the most active chemical properties, and its life is very short.

In the body, OH^- can react with the surrounding molecules and generate level-2 free radicals, so as to destroy cell structure. Studies (Gus'kov et al., 2000; Jin Yue et al.,

2007; Li, 2007a; b) show that some ingredients in some natural plants can effectively clear free radicals, protect body cells and tissues from destruction, effectively resist diseases and delay senility. This experiment explores the clearing effect of LBP on OH^- through Fenton reaction, and the result shows that different concentrations of LBP can clear OH^- radicals. The principle of pyrogallol autooxidation method is to use O_2^+ scavenger to reduce the absorption peak area of pyrogallol autoxidation product at 325 nm and to use ultraviolet spectrophotometer to measure and indirectly calculate the O_2^+ clearance rate for evaluating the antioxidation activity of the test sample. The experimental result shows that LBP has significant effects in clearing O_2^+ radicals, which is consistent with the studies made by other scholars (Meng et al., 2009). This experiment extracts, separates and purifies LBP, to prove the antioxidation activity of LBP.

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Full Length Research Paper

Expression of CD44 in osteoarthritis cartilage tissue and its clinical significance

Bo Liao, Yong Ding, Yong Zhou, Baoan Ma and Yong Zhang*

Department of Orthopaedic Surgery, Tangdu Hospital, The Fourth Military Medical University of PLA, Xi'an 710038, Shaanxi Province, P. R. China.

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The expression of CD44 in osteoarthritis cartilage tissue and its effect in the generation and development of osteoarthritis (OA) were studied. Forty patients with primary OA who received joint arthroplasty were randomly included in this study as OA group. The normal articular cartilages as control group were obtained following Outerbridge standard from twenty patients who received amputation. The cartilages were fixed, decalcified, paraffin embedded and stained with hematoxylin and eosin (HE). Immunohistochemical staining method was used to determine CD44 expression. The fragments were divided into normal group, mild group, moderate group and severe group according to the severity of degeneration. CD44 expressed both in the control and OA group, and mainly localized in the cell membrane. The level of CD44 in OA group was significantly higher than that in the control group ($P<0.05$); the level of CD44 gradually decreased with the severity of OA lesions, that is, mild group > moderate group > severe group, and the three groups had significant differences ($P<0.05$). Pearson correlation analysis showed a significant negative correlation between the CD44 expression levels and the Mankin pathology score in the OA cartilage tissue (r = -6.013, $P<0.01$). Adhesion molecule CD44 highly expressed in osteoarthritis tissue, and gradually decreased with the severity of osteoarthritis lesions.

Key words: Adhesion molecule CD44, osteoarthritis, cartilage tissue, immunohistochemical staining.

INTRODUCTION

Osteoarthritis (OA), also known as osteoarthrosis, degenerative joint disease and senile arthritis, is a common chronic disabling disease. It is also a serious threat to the health of the elderly and can affect their daily activities. It is reported that in the population of 50 years and above, OA is only second to cardiovascular disease among the diseases that lead to long-term disability (Burnett et al., 2006). Commonly, adult articular cartilage regenerative ability is limited, and articular cartilage degeneration seems to be irreversible pathological change. It is generally agreed that causation is multi-factorial, involving age, genetic predetermination, acute and chronic joint trauma, as well as endocrine disorders and, perhaps, dietary factors and local myodynamia (Stove et al., 2006; Li et al., 2009). Due to the lack of effective treatment of OA, the exact pathogenesis is not yet fully elucidated. Therefore, it is particularly important to explore its etiology and pathogenesis to prevent and cure it. CD44 is a transmembrane adhesion molecule, and as a member of the cell surface adhesion molecules, it mainly participates in heterogeneous adhesion. It has been demonstrated that CD44 plays an important role in local clearance of hyaluronan and mediates cell-matrix interactions involved in tumor cells metastasis, tumor formation and T cell extravasation (Liang et al., 2007). A study showed that CD44 is correlated with animal OA

*Corresponding author. E-mail: zhangy7138@gmail.com.

model (Smith et al., 2008). Nevertheless, we did not find any report which showed its relationship with human articular cartilage cataplasia of OA. Therefore, in our present work, the expression of CD44 in OA and different degree retrogression of articular cartilage was measured to reveal the correlation of CD44 with OA, and to further elucidate the mechanism of OA, providing theoretical basis for early diagnosis and treatment for OA.

MATERIALS AND METHODS

According to OA diagnosis standard in “the osteoarthritis treatment guidelines” (2007) revised by Association of Osteology Branch, a total of 40 OA patients who received joint arthroplasty at our hospital between August 2009 and August 2011 were randomly included in the study (Osteoarthritis treatment guidelines, 2007). These patients were all in hospital for primary knee OA, and exclude secondary OA like traumatic OA, and other types of arthritis, such as rheumatoid and suppurative arthritis. They never receive any regular treatment before admission to the hospital. These patients comprised 26 males and 14 females and were average (63.17 ± 11.35) years old (range from 52 to 79 years). Out of these, 27 suffered from hip arthritis and 13 from knee arthritis. Samples were cartilages collected from different lesion location. Correspondingly, 20 normal articular cartilages were collected from knee (patients received amputation because of trauma) or femoral head (patients suffered from femoral neck fracture because of violence) as control group. The onset age of the patients (13 males and 7 females) ranged from 50 to 75 years with average of (61.56 ± 15.89) years. Preoperative X-ray and postoperative clinical diagnosis were conducted to exclude the structural damage specimens, such as degeneration, tuberculosis, infection, tumor, rheumatoid inflammation and marked osteoporosis. And in this study, sex and age were not significantly different in OA and control groups (P>0.05).

Specimen processing

The specimens were obtained following Outerbridge standard from OA group who did joint replacement and control group who received amputation. After fixation with 4% paraformaldehyde and decalcification with buffered EDTA (15% ethylene diamine tetraacetic acid), the tissue samples were dehydrated and embedded in paraffin. Sections (5 μm thick) were cut and stained with HE. The expression of CD44 was investigated with immunohistochemical staining and high-pressure-temperature treatment for antigen retrieval. Of the 50 cartilages, normal group, mild group, moderate group and severe group were divided according to the severity of degeneration guided by the improved Mankin standard (Mankin et al., 1971).

Immunohistochemical staining of CD44

PBS other than primary antibody was used as negative control. The product of immune response was observed. It showed that CD44 protein accumulated on the cell membrane with brown color and fine grain, and nuclei was not stained. 10 regions were selected randomly in the articular cartilage images collected from CD44 immunohistochemical staining slices. Static gray-scale in the MIAS medical image analysis software was used to measure their gray value and the average gray value was used to assess the CD44 expression intensity.

Statistic analysis

Average gray value was applied to analysze the expression differences of CD44 in the four groups and the correlation of CD44 expression with Mankin score. All values were expressed as mean ± SE of mean. Student’s t-test and χ^2 test were used to assess an overall difference among the groups for each of the variables.

Pearson correlation was used for analysis. Probability values less than 0.05 were considered statistically significant (analysis was performed using SPSS for Windows, Version 16.0).

RESULTS

Morphology of cartilage tissue in the control and OA group

Histomorphology observation showed that in the control group, the surface of the cartilage tissue was slightly unsmooth with no crevice and a little bit of proliferation, and the cells were relatively structured. While in the OA group, there were visible erosion and fractures with local fibrosis or all fibrosis. There was disorder in cells proliferation, and there was decrease in the number of cluster or cells, damage in tidal line and there was capillary elapse or disappearance (Figure 1).

Histological grading of OA

The 50 cartilages were classified according to the severity of degeneration guided by the improved Mankin score. The score and group are shown in Table 1.

The expression of CD44 in the control and OA group

The results revealed that adhesion molecule CD44 expressed both in the control and OA groups and mainly concentrated at the cytomembrane (Figure 2). Nonetheless, the level of CD44 in the OA group was significantly higher than that in the control group (P<0.05); the concentration of CD44 gradually declined with the severity of OA, that is, mild group > moderate group > severe group, and the three groups have significant difference (P<0.05) (Table 2).

The correlation of the CD44 levels in OA cartilage tissues with Mankin score

Pearson correlation analysis indicated that CD44 level in the OA group was negatively correlated with Mankin pathologic score (r =-6.013, P<0.01).

DISCUSSION

Articular cartilage degenerative change, bone reactive hyperplasia of the joint edge and subchondral bone are

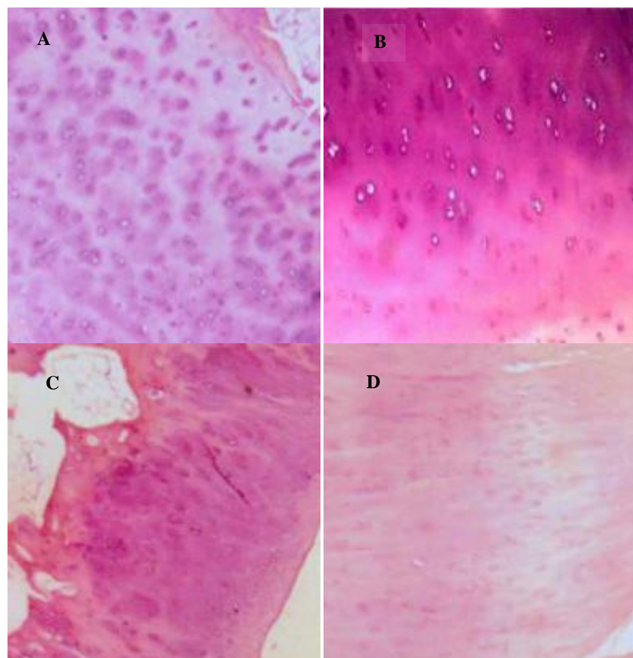

Figure 1. Morphological observations of cartilaginous tissue in the control and OA group (stained with HE, 100×). (A: In the control group, the surface was smooth, and cells were orderly arranged and evenly distributed; B: In the mild group, the surface was slightly unsmooth, and cells were irregularly arranged and unevenly distributed; C: In the moderate group, the surface was fibrosis, and cells were disarray in clusters with incomplete tidal line; D: In the severe group, the surface was fibrosis, and the numbers of cells were decreased obviously).

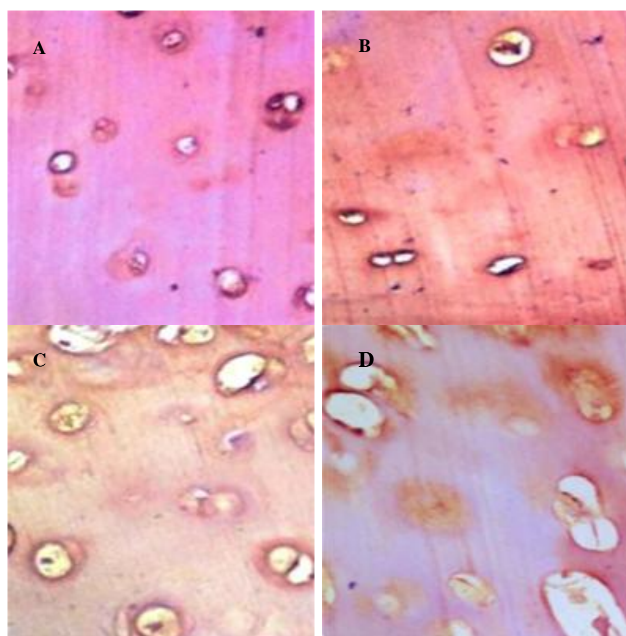

Figure 2. CD44 expressions in the control and OA group (stained with HE, 400×). Groups A, B, C and D show the expression of CD44 in the control, mild, moderate and severe OA groups, respectively.

Table 1. Mankin score.

Group	No.	Mankin score
Control	20	0.57±0.19
Mild	11	3.38±1.13
Moderate	13	7.18±2.39
Severe	16	12.61±4.20

Table 2. Gray value of CD44 in the control and OA group ($\bar{x} \pm s$).

Group	No.	CD44
Control	20	181.17
OA	50	140.60*
Mild	11	165.33*
Moderate	13	146.52$^{*\#}$
Severe	16	118.80$^{*\#\blacktriangle}$

*Comparison of the value in the OA group with control group: *P<0.05. Comparison of the value in the OA group with mild group: $^{\#}P$<0.05 and with moderate group: $^{\blacktriangle}P$<0.05.

the main pathological changes of OA. Here, series of changes have taken place in the articular cartilage, synovium and synovial cartilage, collagen, proteoglycan as well as histocyte. However, the mechanism of its pathogenesis is not yet fully elucidated presently, especially the understanding of the joint cartilage degeneration in cell and molecular field. Therefore, to explore the pathogenesis of OA at cell and molecular level would help to make clearer, the clinical significance of its prevention and treatment.

CD44 is a kind of stranded cell surface-glycoprotein (GP) encoded by a single gene, which is widely present on the membrane of blood cells, fibroblasts, epithelial cells and endothelial cells (Lou et al., 1999). CD44 gene is also a member of the cell surface adhesion molecules that is encoded by 20 highly conservative exons and can be classified as constitutive (c) and variant (v) isoform by different transcription of the 20 exons. That is, the constitutive type called CD44s, has 10 exons and the transcription segment exists in all CD44 transcripts; another one is alternative spliced by 10 variant exons, called CD44v. Extracellular matrix components have been described as ligands for the CD44, such as hyaluronic acid (HA), fibronectin and collagen. And functional diversity of CD44 seems to be influenced by the multiple natures of ligands (Naor and Nedvetzki., 2003). To sum up, the functions of CD44 including (Chel et al., 2006; Mckallip et al., 2002): homing lymphocytes rolling on the vessels through mediating adhesion of lymphocytes to high endothelial venules; involving in lymphocyte mediation; combining with HA and laminin (LN); binding to cytoskeleton protein and participating in formation of locomotion cells and CD44 which has been

shown to have a crucial role in the cell migration.

Research by Weber et al. (2002) showed that in an induced OA rabbit model, CD44 was highly expressed in different stages of the cartilage by immunohistochemical staining, and the level was associated with histological score criteria. Nedvetzke et al. (1999) injecting anti-CD44 mAbs into type II collagen-induced OA mice showed that anti-CD44 mAb markedly reduced the synovial inflammatory cellular response and the consequent damage to the joint. In addition, eighteen sheep had bilateral lateral meniscectomy to induce OA and sections of synovium were immunostained. Smith et al. (2008) found that CD44 was increased in the synovial lining of OA joints, and HA treatment reduced features of the pathology and improved joint mobility and function in OA. Furthermore, clinical research (Tibesku et al., 2006) revealed that in the serum and synovium, CD44 expression in OA patient was markedly up-regulated than that in healthy subjects. CD44, known to be the principle cell surface receptor for HA, plays a vital role in the extracellular matrix components metabolism, especially HA metabolism.

This study used immunohistochemical staining to determine CD44 concentration in the normal and OA cartilage tissue. Results showed that the level of CD44 in OA group was significantly higher than that in the control group, and there were significant differences between them (P<0.05); the level of CD44 gradually decreased with the severity of OA lesions, that is, mild group > moderate group > severe group, and the three groups were significantly different (P<0.05). The findings suggested that CD44 could promote the development of articular cartilage lesion of OA, and the possible mechanism may be that highly CD44 expression enhances endocytosis and the capacity of its binding to HA in the extracellular cartilage matrix. Thus, the HA's decomposition finally results to imbalance of extracellular cartilage matrix metabolism, which accelerates cartilage degradation. We also found a significant negative correlation between the CD44 expression levels and the Mankin pathology score in the OA cartilage tissue (r = -6.013, P<0.01), which showed that CD44 gradually decreased with the severity of OA.

In conclusion, adhesion molecule CD44 is highly expressed in cartilage tissue of OA, and shows a gradual decline with the severity of OA, which provide some clue to further study of the pathogenesis of OA. However, the complexity of OA pathogenesis should be noticed. It is not enough to represent OA progression only by the articular cartilage degeneration degree which cannot reflect the comprehensive pathological process of OA. Moreover, the mechanism of CD44 is not certain at present. Consequently, further studies are needed.

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Full Length Research Paper

Efficacy of different medicines used for the treatment of osteoporosis by using dual energy x-ray absorptiometry

Perveen Zaidi, Muhammad Hanif*, Shahid Kamal, Salman Habib and Akhtar Ahmed

Karachi Institute of Radiotherapy and Nuclear Medicine, Pakistan.

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Osteoporosis, a skeletal disease and common condition affecting one in three women and one in twelve men is a major health burden worldwide and in our population as well. A total of 180 patients including 30 in control group, 126 osteoporotics and 54 osteopenics, were diagnosed and analyzed with the help of bone mineral density (BMD) by dual energy x-ray absorptiometry (DEXA) and treated in different groups with different brands of medicines; bisphophonates (alendronates and risederonates). Overall results in the therapy group BMD (g/cm²) spine improved from 0.748 ± 0.0088 to 0.777 ± 0.0091 after one year of treatment while BMD hip rose from 0.713 ± 0.0087 to 0.730 ± 0.009 in a similar period. In the osteoporotic group (n = 106), BMD spine increased from 0.699 ± 0.0077 to 0.727 ± 0.007 and BMD hip from 0.679 ± 0.009 to 0.693 ± 0.009. In the osteopenic goup (n = 44), BMD spine increased from 0.863 ± 0.011 to 0.898 ± 0.011 and BMD hip from 0.793 ± 0.007 to 0.817 ± 0.012. Patients on Osto, Drate and Fosamax (alendronates) did better than those on Dronate and Actonel (risedronates). Of alendronates, Fosamax and Osto treated patients did better than those on Drate. Of risedronates, Actonel treated patients faired better than those on Dronate which showed the least improvement.

Key words: Osteoporosis, osteopenia, bone mineral density, DEXA, alendronate, risedronate.

INTRODUCTION

Osteoporosis is a skeletal disease characterized by low bone mass and micro architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. Osteoporosis is a common condition affecting one in three women and one in 12 men, resulting in a substantial morbidity, excess mortality, health and social services expenditure (Nelson et al., 2002). The WHO definition of osteoporosis is based on measurement of bone mineral density (BMD) of >2.5 standard deviations (SD) below the mean for young adults, while osteopenia is defined as a BMD between 1 and 2.5 SDs below the means for young adults (T score) (Estell, 1998) (WHO report, 1994). The risk of fracture increases to three fold for each SD decrease in BMD (Martial et al., 1996). The disease is common in postmenopausal women (Melton et al., 1990); however, the disease prevalence varies in different population. Umer et al. (2003) reported the prevalence of osteoporosis 8.7% and osteopenia 22.5% in postmenopausal in Mayo hospital Lahore. In another mega study, 40% postmenopausal osteopenia and 7% osteoporosis was found by peripheral bone densitometry (Siris et al., 2001). It is widely accepted that BMD measurement using DEXA is the gold standard of diagnosis for osteoporosis (Siris et al., 2001; Lewiecki et al., 2004; Grampp et al., 1999). A study conducted by Habiba et al. (2002) at Hayatabad

*Corresponding author. E-mail: hanifmuhammad@hotmail.com.

Medical Complex, Peshawar in 1997 to 1998 on thousand postmenopausal women for simple calculated osteoporosis risk estimation, found that 75.3% were predisposed to osteoporosis and the risk increased with age (97% in women of 75 to 84 years of age compared to 55% in women of 45 to 54 years of age). The importance of developing treatments that reduce the risk of fracture is evident, both from an individual and a societal perspective, and a number of agents are available that have been shown in randomized controlled trials to decrease the risk of vertebral and, in some instances, non-vertebral fracture (Delmas, 2002; Compston et al., 2009). Major pharmacological interventions are the bisphosphonates, strontium ranelate, raloxifene, denosumab and parathyroid hormone peptides. They are approved only for the treatment of postmenopausal osteoporosis, but alendronate, etidronate, risedronate and zoledronic acid are also approved for the prevention and treatment of glucocorticoid-induced osteoporosis (Van Staa, 2006; Compston, 2007) and alendronate, risedronate, zoledronate and teriparatide are approved for the treatment of osteoporosis in men (Papaioannou et al., 2010; Compston et al., 2009).

The alendronate is USFDA approved for the prevention and treatment of osteoporosis in postmenopausal women. It is also approved as a treatment to increase bone mass in men with osteoporosis and glucocorticoid induced osteoporosis in both men and women.

Alendronate prevents osteoporosis in post-menopausal women (McClung et al., 1998). Alendronate reduces new vertebral and non-vertebral fractures during treatment of osteoporosis (Black et al., 1996). The effects of alendronate in osteoporotic men appear similar to those seen in postmenopausal women (Orwoll et al., 2000). There is significant change in BMD in patients using long term prednisolone therapy (Saag et al., 1998). The 5 mg daily dose and 35 mg weekly dose have been approved by the FDA for prevention of postmenopausal osteoporosis, and the 10 mg daily dose and 70 mg weekly dose have been approved for treatment in men and postmenopausal women (Peters et al., 2001).

Risedronate was recently approved for treatment of osteoporosis. The results appear similar to alendronate. Risedronate significantly reduces the risk of hip fracture among elderly women with confirmed osteoporosis but not among elderly women selected primarily on the basis of risk factors other than low bone mineral density. There are conflicting data about whether more gastrointestinal side effects are seen with alendronate than with risedronate. At this time, this newer bisphosphonate does not seem to provide any definite advantage to alendronate for the treatment of osteoporosis, other than possibly price. Risedronate prevents osteoporosis in post-menopausal women (Hooper et al., 1999; Fogelman et al., 2000). Alendronate reduces new vertebral and non-vertebral fractures during treatment of osteoporosis (Reginster et al., 2000; Harris et al., 1999). There is significant change in BMD in patients using long term prednisone therapy (Cohen et al., 1999; Reid et al., 2000). For treatment in postmenopausal women and for treatment in perimenopausal women (35 mg tablet once a week or 5 mg tablet once daily). For treatment and prevention in men and women 5 mg tablet once daily. It has been demonstrated in studies to increase bone mass in the spine and hip and reduce the risk of spine and non-spine fractures by 40 to 50% over a 3 to 5 year period. 30 mg is given once daily for 2 months. It is a USFDA approved drug for treatment of Paget's disease (Peters et al., 2001).

According to our knowledge, not much work has been done so far on osteoporosis in particular on efficacy of different medicines and BMD. The objective of the study was to find out the efficacy of different medicines with the help of bone mineral density (BMD) measured by latest technique of dual energy X-ray absorptiometery (DEXA) for the diagnosis and treatment of osteoporosis in pre and postmenopausal women of Karachi.

MATERIALS AND METHODS

Setting

The study was conducted for a period of two years and each patient studied (treated and analyzed) for one year in patients attending the outpatient department of Karachi Institute of Radiotherapy and Nuclear Medicine, Karachi.

Sample size and characteristics

This study was carried out on 180 postmenopausal women, belonging to the urban population of Karachi. Out of those, 30 subjects were placed in the control or placebo group while 150 patients of osteoporosis/osteopenia underwent therapy. Patients undergoing therapy were further subdivided into five sub-groups of 30 each; depending on the drug treatment they received, that is, Osto, Drate, Fosamax, Dronate or Actonel.

On the basis of BMD values, patients were diagnosed as having osteoporosis or osteopenia and their data was calculated on a quarterly basis. The "term baseline" or "0 month" refers to the time when patients were initially admitted and treatment initiated. Similarly 3, 6, 9 and 12 months refer to the length of time of treatment from baseline or 0 month.

Patient selection

After considering the detailed history taken from patients, the population is selected for the study in which no family history of osteoporosis, thyroid disorder and other factors observed which may affect the bone mineral density.

Detailed history from the patient was taken concerning previous drug, surgical, medical, gynaecological history, and if any test has been performed previously. The detailed performance was also filled by the patient.

Design

Bisphosphonates (Alendronate and Risedronate) were prescribed to the patients under different brand names according to the indicated dosage. The primary end point was the change in bone

mineral density at the hip and spine. Bone mineral density was measured quarterly (0, 3. 6, 9 and 12 months) by dual-energy x-ray absorptiometry in a blinded fashion.

Dosage

The usual dosing recommendations are according to Peters et al. (2001). In our study, the dosage was:

1. Alendronate
(a) FOSAMAX 70 mg weekly
(b) DRATE 70 mg weekly
(c) OSTO 70 mg daily

2. Risedronate
(a) DRONATE 35 mg weekly
(b) ACTONEL 35 mg weekly

The drug is to be taken only upon rising for the day with three swallows of water, not to exceed 6 to 8 oz. Stand, walk or sit and remain fasting for 30 to 45 min afterwards, then take breakfast. Lying down or reclining prior to taking breakfast may cause gastroesophageal reflux and esophageal irritation. At least 30 min should be allowed to pass before meals or other beverages than water is taken in.

i) Alendronate and risedronate are generally well tolerated as long as they are taken appropriately to avoid upper gastrointestinal adverse effects.
ii) Alendronate is slightly more expensive than risedronate; however, the once-weekly form of alendronate may enhance patient compliance and tolerability enough to offset the higher cost.
iii) Alendronate is not FDA-approved for preventing glucocorticoid induced osteoporosis.

Side effects

1. GI tract: A severe side effect is an ulceration of the esophagus caused by alendronate, which may require hospitalization and intensive treatment. Gastric and duodenal ulceration.
2. General: Infrequent cases of skin rash, rarely manifesting as Stevens-Johnson syndrome and toxic epidermal necrolysis, eye problems (uveitis, scleritis) and generalized muscle, joint, and bone pain (rarely severe) have been seen. In laboratory tests, decreased calcium and phosphate values may be obtained but reflect action of the drug and are harmless.
3. Cases of osteonecrosis of the jaw have been reported in the medical literature, but relationship to alendronate is unknown.
4. Osteonecrosis of the jaw-deterioration of the TM joint can also result specially in cancer patients.
5. Rare instances of auditory hallucinations and visual disturbances have been associated with alendronate and other bisphosphonates.

DEXA

Bone mineral density was measured by latest technique of DEXA. BMD was measured by DXA with a QDR (quantitative digital radiography) discovery device (Hologic, Waltham, MA, USA) at the lumber spine (LS BMD) and total hip (H BMD) at KIRAN, Karachi.

Statistical analysis

Statistical analysis of this study was carried out using, SPSS for windows version 12.0 and MedCalc® version 9.5.2.0. Variables for SPSS were defined according to the parameters listed in patient data form. Data was entered with sequence represent in patient data form for each treatment group and finally of control group. Least significant change of the system was calculated using spine phantom. By taking LSC = 2% for both Hip and Spine ROC, curves were obtained using MedCalc® version 9.5.2.0. Corresponding cut-offs and positive predictive values (PPV), negative predictive values (NPV), sensitivity and specificity were also calculated at 3, 6, 9 and 12 months of overall population (except control group). Correlation is significant at the 0.01 level (2-tailed). All aforementioned parameters were also calculated on treatment group basis.

RESULTS

Table 1 shows the characteristics of the population of this study. Table 2 shows the comparison of biophysical parameters of the therapy and control groups.

In the therapy group, BMD (g/cm²) spine improved from 0.748 ± 0.0088 to 0.777 ± 0.0091 after one year of treatment while BMD hip rose from 0.713 ± 0.0087 to 0.730 ± 0.009 in a similar period (Table 3). The values are expressed as mean ± standard error (SE) in mean. Table 3 shows the average BMD spine and BMD hip of all the patients (n = 150) in the therapy group at baseline and at 3, 6, 9 and 12 months. The values are expressed as mean ± standard error (SE) in mean

In the osteoporotic group (n = 106), BMD spine increased from 0.699 ± 0.0077 to 0.727 ± 0.007 and BMD hip from 0.679 ± 0.009 to 0.693 ± 0.009. In the osteopenic group (n = 44), BMD spine increased from 0.863 ± 0.011 to 0.898 ± 0.011 and BMD hip from 0.793 ± 0.007 to 0.817 ± 0.012 (Table 4). The values are expressed as mean ± standard error (SE) in mean. In Table 4, data of BMD spine and hip is given based on the sub-classification of patients into osteoporosis (n = 106) and osteopenia (n = 44) groups.

The five therapy sub-groups behaved much differently than the control group. In the control group, BMD fell from 0.757 ± 0.014 to 0.741 ± 0.014 in case of spine and from 0.729 ± 0.017 to 0.719 ± 0.017 in case of hip, over a one year period (Table 5). In Table 5 data of BMD spine in therapy and control groups against treatment time is shown. The values are expressed as mean ± standard error (SE) in mean.

In case of Osto, Drate, Actonel and Fosmax, it rose steadily for spine (0.786 ± 0.023 to 0.827 ± 0.025, 0.734 ± 0.017 to 0.764 ± 0.018, 0.736 ± 0.017 to 0.763 ± 0.015, and 0.739 ± 0.019 to 0.774 ± 0.020, respectively) (Table 5) and hip (0.734 ± 0.023 to 0.761 ± 0.024, 0.715 ± 0.021 to 0.730 ± 0.022, 0.692 ± 0.015 to 0.708 ± 0.015 and 0.722 ± 0.019 to 0.746 ± 0.02, respectively) (Table 6).

However, a somewhat different pattern was noted in case of Dronate) (Tables 5 and 6). In Table 6 data of BMD hip in therapy and control groups against treatment time is shown. The values are expressed as mean ± standard error (SE) in mean.

After an initial rise till six months, a subsequent drop was noted. It can be appreciated that the average BMD in osteoporosis group is less than that in osteopenia group because of greater bone loss in former.

Table 1. Comparison of biophysical parameters of therapy and control groups.

Parameter	Therapy group	Control group
Total no of subjects	150	30
Patients with osteoporosis	106	20
Patients with osteopenia	44	10
Mean age (years)	55.10	55.60
Age range (years)	37 - 76	46 - 69
Mean Weight (kg)	60.86	61.26
Weight range (kg)	35 - 85	39 - 76
Average time since menopause in osteoporosis group (years)	10.67	8.15
Average time since menopause in osteopenia group (years)	8.50	9.40

Table 2. Demographic data of sub-groups of the study population.

Group	Osteoporosis			Osteopenia		
	Mean age (years)	Mean height (cm)	Mean weight (kg)	Mean age (years)	Mean height (cm)	Mean weight (kg)
Osto	56 ± 8	55 ± 10	149 ± 3	53 ± 7	68 ± 7	153 ± 3
Drate	56 ± 6	57 ± 8	150 ± 4	54 ± 11	65 ± 7	158 ± 11
Dronate	56 ± 6	58 ± 10	150 ± 7	49 ± 10	66 ± 10	150 ± 7
Actonel	53 ± 7	57 ± 7	152 ± 7	55 ± 9	65 ± 8	154 ± 5
Fosamex	57 ± 7	58 ± 10	152 ± 6	54 ± 7	74 ± 5	156 ± 4
Control	55 ± 5	58 ± 8	150 ± 6	55 ± 5	66 ± 6	151 ± 7
Total	55 ± 6	57 ± 9	151 ± 5	53 ± 8	68 ± 7	153 ± 6

Table 3. Average BMD of spine and hip of all patients in the therapy group (improvement of spine and hip BMD with therapy)

Time (months)	BMD spine	BMD hip
0	0.748 ± 0.0088	0.713 ± 0.0087
3	0.763 ± 0.0087	0.717 ± 0.0088
6	0.768 ± 0.0089	0.723 ± 0.0087
9	0.772 ± 0.0090	0.726 ± 0.0088
12	0.777 ± 0.0091	0.730 ± 0.009

Table 4. Comparison of BMD spine and hip in osteoporotic and osteopenic patients after treatment.

Time (months)	BMD spine		BMD hip	
	Osteoporosis (n = 106)	Osteopenia (n = 44)	Osteoporosis (n = 106)	Osteopenia (n = 44)
0	0.699 ± 0.0077	0.863 ± 0.011	0.679 ± 0.009	0.793 ± 0.0077
3	0.717 ± 0.008	0.873 ± 0.011	0.683 ± 0.009	0.799 ± 0.008
6	0.720 ± 0.008	0.883 ± 0.011	0.689 ± 0.009	0.804 ± 0.008
9	0.723 ± 0.008	0.889 ± 0.011	0.691 ± 0.009	0.810 ± 0.008
12	0.727 ± 0.007	0.898 ± 0.011	0.693 ± 0.009	0.817 ± 0.012

In terms of percentage change in BMD after one year, the groups behaved variably. Osto treated patients showed an improvement in BMD of 5.21% for spine and 3.67% for hip at 12 months. In case of Drate, it was 4.08% for spine and 2.11% for hip. For Actonel, it was 3.66% for spine and 2.31% for hip. In case of Fosamax,

Table 5. BMD spine in therapy and control groups against treatment time.

Time (months)	Osto	Drate	Dronate	Actonel	Fosamax	Control
0	0.786 ± 0.023	0.734 ± 0.017	0.743 ± 0.022	0.736 ± 0.017	0.739 ± 0.019	0.757 ± 0.014
3	0.799 ± 0.023	0.752 ± 0.017	0.762 ± 0.021	0.751 ± 0.016	0.752 ± 0.018	0.753 ± 0.014
6	0.810 ± 0.024	0.756 ± 0.018	0.762 ± 0.021	0.756 ± 0.016	0.759 ± 0.019	0.749 ± 0.014
9	0.818 ± 0.025	0.760 ± 0.018	0.757 ± 0.021	0.758 ± 0.015	0.766 ± 0.019	0.745 ± 0.014
12	0.827 ± 0.025	0.764 ± 0.018	0.755 ± 0.021	0.763 ± 0.015	0.774 ± 0.020	0.741 ± 0.014

Table 6. BMD hip in therapy and control groups against treatment time.

Time (months)	Osto	Drate	Dronate	Actonel	Fosamax	Control
0	0.734 ± 0.023	0.715 ± 0.021	0.699 ± 0.017	0.692 ± 0.015	0.722 ± 0.019	0.729 ± 0.017
3	0.742 ± 0.023	0.716 ± 0.020	0.704 ± 0.017	0.696 ± 0.015	0.728 ± 0.019	0.726 ± 0.017
6	0.746 ± 0.023	0.722 ± 0.021	0.707 ± 0.0170	0.701 ± 0.015	0.739 ± 0.019	0.724 ± 0.017
9	0.751 ± 0.023	0.726 ± 0.021	0.705 ± 0.017	0.704 ± 0.015	0.744 ± 0.02	0.721 ± 0.017
12	0.761 ± 0.024	0.7301 ± 0.022	0.703 ± 0.017	0.708 ± 0.015	0.746 ± 0.02	0.719 ± 0.017

Table 7. Percentage change in BMD spine in therapy and control groups.

Time (months)	Percentage change in BMD spine from baseline (%)					
	Osto	Drate	Dronate	Actonel	Fosamax	Control
3	1.65	2.45	2.55	2.03	1.75	-0.52
6	3.05	2.99	2.55	2.71	2.70	-1.05
9	4.07	3.54	1.88	2.98	3.65	-1.58
12	5.21	4.08	1.61	3.66	4.73	-2.11

Table 8. Percentage change in BMD hip in therapy and control groups.

Time (months)	Percent change in BMD Hip from baseline					
	Osto	Drate	Dronate	Actonel	Fosamax	Control
3	1.089	0.13	0.71	0.57	0.83	-0.41
6	1.63	0.97	1.14	1.30	2.35	-0.68
9	2.31	1.53	0.85	1.73	3.04	-1.09
12	3.67	2.11	0.57	2.31	3.32	-1.37

spine showed 4.73% while hip showed 3.32% improvement at one year. In case of Dronate, spine improved by 2.55% at six months but at 12 months it fell to 1.61%; for hip, it increased by 1.14% at 6 months to drop to only a 0.57% increment at 12 months (Tables 7 and 8).

Patients on Osto, Drate, Fosamax (alendronates) did better than those on Dronate and Actonel (risedronates). Of alendronates, Fosamax and Osto treated patients did better than those on Drate. Of risedronates, Actonel treated patients faired better than those on Dronate which showed the least improvement.

In order to get a better understanding about the improvement in BMD, spine and hip in the five therapy subgroups, the percentage change from baseline was calculated and is given in Tables 7 and 8, and show how the percentage change in BMD spine and hip vary for therapy and control groups. In the group of untreated patients (control), BMD continuously drops so percentage change lies in negative region. Response in case of Osto and Fosamax is quite good throughout the treatment time while it is somewhat slower in case of Drate after 6 months. Much slower improvement is observed in case of Actonel. In case of Dronate, a rise at 3 months and then a continuous drop is observed throughout the treatment course (Tables 7 and 8).

DISCUSSIONS

The increasing awareness of osteoporosis and the development of treatments with proven efficacy are likely to increase the demand for management and monitoring of patients with osteoporosis. This in turn will require widespread facilities for its diagnosis and assessment. Measurements of bone mineral density are a central component since this forms an integral component of the definition of osteoporosis. The internationally agreed description of osteoporosis is 'a systematic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures'. The definition captures the notion that low bone mineral density is an important component of the risk of fracture, but recognizes that other abnormalities in the skeleton contribute to skeletal fragility.

Although the measurement of BMD by DEXA is accepted as the 'gold standard' for establishing the diagnosis of and for follow-up of patients with osteoporosis/osteopenia, it must be emphasized here that sources of potential limitations in the accuracy of the technique must be kept in mind. These may accrue from systematic inaccuracies, biological variability, variable soft tissue densities, site related inaccuracies etc. This highlights the need to establish the performance characteristic of any technique for any site by establishing its sensitivity, specificity, and positive predictive value (Hui, 1988).

The importance of developing treatments against osteoporosis and osteopenia is evident, both from an individual and a societal perspective, and a number of agents are available that have been shown in randomized controlled trials to decrease the risk of vertebral and, in some instances, non-vertebral fracture (Delmas, 2002; Compston, 2009).

Major pharmacological interventions are the bisphosphonates, strontium ranelate, raloxifene, denosumab and parathyroid hormone peptides. Interventions that are approved for the prevention and treatment of osteoporosis in Europe are approved only for the treatment of postmenopausal osteoporosis, but alendronate, etidronate, risedronate and zoledronic acid are also approved for the prevention and treatment of glucocorticoid-induced osteoporosis (Van Staa, 2006; Compston, 2007) and alendronate, risedronate, zoledronate and teriparatide are approved for the treatment of osteoporosis in men (Compston, 2009; Papaioannou, 2010).

A total of 180 patients were included in this study. Out of these, 150 underwent therapy while 30 did not take medication for various reasons like alternate treatment by homeopaths, hakims, diet treatment or other reasons for non-compliance and served as the control group for the study. Patients were sub-classified on the basis of BMD measurements by DXA into osteoporotic (T score < -2.5 SD at any site) and osteopenic (T score between -1.5 to -2.5 SD at any site) groups. Of the 150 patients undergoing therapy (mean age 55.10 years, mean weight 60.86 kg), 106 patients belonged to the osteoporotic group while 44 belonged to the osteopenic group. In the control group (30 patients), 20 were osteporotic while 10 were osteopenic. The therapy group was further sub-divided into 5 groups of 30 patients each depending on the drug treatment given, that is, Osto, Drate, Fosamax (alendronates) or Dronate and Actonel (risedronates).

Data analysis shows after 12 months significantly higher average BMD increases at lumber spine and at total hip for patients treated either with alendronates or risedronates, although five therapy sub groups behaved much differently than the control group.

Although our results contrast with findings from other observational studies that document risedronate as more effective than alendronate in preventing nonvertebral fractures (Watts, 2004; Silverman, 2007), it is also somewhat surprising because randomized, controlled trials (RCTs) show that alendronate improves bone mineral density and reduces bone turnover markers better than risedronate (Rosen, 2005; Bonnick, 2006). Previous studies comparing bisphosphonates included preventive doses of alendronate that are less effective than treatment doses (Cranney, 2002**)**. These differences in study may partially explain the differences between our findings between bisphosphonates, compared with previous studies suggesting that risedronate is more effective than alendronate.

To our knowledge, FACT (Fosamax Actonel Comparison Trial) is the only head-to-head trial comparing alendronate and risedronate (Rosen, 2005; Bonnick, 2006) randomly assigning 1053 postmenopausal women (mean age, 64.5 years) with low bone mineral density to receive weekly alendronate or risedronate, FACT controlled for both measured and unmeasured confounding. However, FACT also excluded important candidate groups for pharmacotherapy with bisphosphonates, such as men, and women with previous hormone or long-term glucocorticoid therapy. Randomized, controlled trials establish drug efficacy within defined patient populations that are often not representative of those who may benefit from pharmacotherapy or of how the agents are used in practice (for example, adherence to drug regimen, and calcium or vitamin D supplementation) (Lindsay, 2007). In contrast, health care claims data reflect routine practice for large and representative populations (Schneeweiss, 2005**.** Therefore, observational studies play an important role in examining drug effectiveness among those treated. Although alendronate and risedronate recipients in our study were similar according to measured covariates, we cannot rule out possible differences due to unmeasured variables, such as bone mineral density, risk for falls, family history, or nonprescription preventive therapies.

The efficacy of bisphosphonates in reducing

nonvertebral fracture risk is established among persons with a bone mineral density T-score less than −2.5. However, the National Osteoporosis Foundation recommends that treatment be considered at a T-score less than −2.0, and in the presence of other risk factors, at a T-score less than −1.5 (National Osteoporosis Foundation, 2003). It is therefore possible that a high proportion of recipients have a bone mineral density higher than that for which bisphosphonates are documented to be effective. These analyses suggested that our findings are unlikely to be entirely due to unmeasured confounding. Bone mineral density is the most important risk factor for fracture that was not included in our analysis. The relative risk for hip fracture is estimated to be 2.5 at age 65 years among persons with osteoporosis (T-score < –2.5) compared with those with higher bone mineral density (Black, 2006).

The study cohort was limited to low-income families with complete drug coverage residing in Karachi. Thus, our results may not be generalizable to all recipients of these agents, particularly if adherence to treatment differs among those with different drug coverage. However, our cohort of frail persons age 65 years or older is typical of patients requiring pharmacotherapy to reduce fracture risk and provides real-world comparative effectiveness data among patients with complete drug coverage.

In the absence of RCT evidence, observational data provide a complementary source of information that compares drug effectiveness when prescribed in clinical practice (Lindsay, 2007). Our large observational study of persons age 65 years or older who received drug treatment for osteoporosis, identified no difference in the effectiveness of bisphosphonates (risedronate versus alendronate) in preventing nonvertebral fractures. We also documented no large differences in fracture risk among raloxifene compared with alendronate. However, confidence bounds were wide and thus do not rule out potentially important clinical differences.

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Short Communication

Synthesis of three formamidine disulphide derivatives as potential integrase inhibitors of human immunodeficiency virus (HIV)

Afolabi Ezekiel O.* and Jegede Abiodun O.

Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, University of Jos, P.M.B. 2084, Jos, Plateau State, Nigeria.

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Three analogues of formamidine disulphide (FMD) were synthesized as potential integrase inhibitor of human immunodeficiency virus (HIV). Piperonal, 3-Nitrobenz-aldehdye and 3,4,5-trimethoxybenzaldehyde, were respectively coupled with formamidine disulphide to prepare piperoformamidine disulphide (P-FMD), 3-Nitro-formamidine disulphide (N-FMD) and 3,4,5-trimethoxyformamidine disulphide (T-FMD). The structures of the three products were confirmed with infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectroscopic techniques, as reported.

Key words: Human immunodeficiency virus (HIV), acquired immune deficiency syndrome (AIDS), formamidine disulphide (FMD).

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is a set of symptom and infections resulting from damage to the human immune system caused by human immuno-deficiency virus (HIV) (Weiss, 1993). After years of hard work, a number of reverse transcriptase and protease inhibitors were discovered and introduced into clinical practice (Roberto et al., 2003). A new therapeutic target however is the integrase enzyme (Angelo and Mouscadet, 2001). Integrase inhibitors have been shown to display synergy when used in combination with reverse transcriptase and protease inhibitors (Matte, 2001) and has helped to reduce incidence of resistance due to monotherapy (Stephenson, 2007). Many classes of compounds have been shown to inhibit the HIV-1 integrase enzyme. These include DNA binders, peptides, oligonucleotides, nucleotides and polyhydroxylated aromatics (Pani, 2000). Studies have shown that disulphiram (An alcohol dehydrogenase inhibitor is some worth beneficial to victims of AIDS).

A similarity between the alcohol dehydrogenase and the HIV viral integrase was proposed by AHFS-Drug Info, 2007. Thus, it is possible to synthesis HIV integrase inhibitors with similar spatial arrangement to disulfram. This line of thought led to the synthesis of formamidine disulphide derivatives as potential integrase inhibitor of HIV.

METHODOLOGY

Typical synthesis of formamidine disulphide derivative (Scheme I)

A solution of 12 g of thiourea in 40 ml of concentrated H_2SO_4 was added to an acidified $KMnO_4$ until the purple colour disappeared. The excess acid was then neutralized to blue litmus paper with $NaHCO_3$. The formamidine disulphide (FMD) precipitated was filtered and air dried. The desired aldehyde (0.025 M) was added to a solution of FMD weighing 2 g (0.0133 M) in 5 ml of CH_3OH. The

*Corresponding author. E-mail: afolabie@unijos.edu.ng. Tel: +234 (0) 803 5889 579.

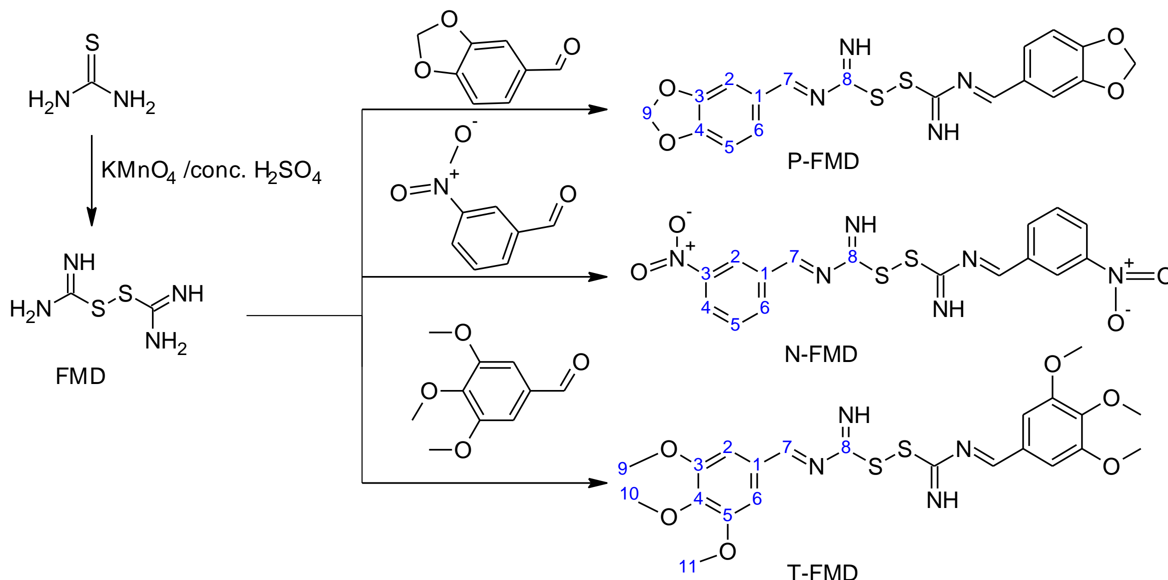

Figure 1. Synthesis of formamidine disulphide derivatives.

mixture was warmed on a water bath for 0.25 h with continuous stirring. On cooling, the crystals formed were filtered and air dried. Piperonal, 3-Nitrobenzaldehdye and 3,4,5-trimethoxybenzaldehyde, were used, respectively to prepare piperoformamidine disulphide (P-FMD), 3-Nitroformamidine disulphide (N-FMD) and 3,4,5-trimethoxyformamidine disulphide (T-FMD).

RESULTS

Spectral analyses of piperoformamidine disulphide

Infrared (IR) (Vmax): 750, 1100, 1250, 1050, 1960, 2350 cm^{-1}; 1H nuclear magnetic resonance (NMR) ($CDCl_3$), δ: 6.9[2H, s], 7.32[1H, d], 7.38[1H, s], 7.41[1H, d] and 9.8[2H, s] correspond with C-9, C-6, C-2, C-5 and C-7; 13C NMR ($CDCl_3$), δ: 190.2, 153.1, 148.7, 131.9, 128.6, 108.3, 106.9 and 102.1 correspond with C-7,8, C-3, C-4, C-1, C-6, C-5, C-6 and C-9, respectively; mass spectrometry (MS) fragmentation pattern: 414 m/z[M^+], 196, 181, 150, 149 [base peak], 125, 121, 110, 77, 68, 91 and 63 m/z.

Spectral analyses of 3-Nitroformamidine disulphide

IR (V max): 610, 720, 800, 1350, 1380, 1550, 1620 and 2350 cm^{-1}; 1H NMR ($CDCl_3$), δ: 7.78[1H, t], 8.2[1H, d], 8.4[1H, d], 8.7[1H, s] and 10.13[1H, s] correspond with C-5, C-6, C-4, C-2 and C-7; 13C NMR ($CDCl_3$), δ: 189.7, 148.8, 137.4, 134.7, 130.4, 128.5 and 124.3 correspond with C-7,8, C-3, C-2, C-1, C-4, C-6 and C-5, respectively; MS fragmentation pattern [M^+][Metastable]: 150, 121 [base peak], 105, 92, 83 and 77m/z.

Spectral analyses of 3,4,5-trimethoxyformamidine disulphide

IR (V max): 680, 1100, 1300, 1650 and 2350 cm^{-1}; 1H NMR ($CDCl_3$), δ: 3.92[9H, s], 7.12[2H, s] and 9.85[1H, s] correspond with C-9, 10, 11, C-2, 6 and C-7; 13C NMR ($CDCl_3$), δ: 190.9, 153.6, 143.7, 131.7, 106.8, 60.9 and 56.3 correspond with C-7,8, C-3,5, C-4, C-1, C-2,6, C-10 and C-9,11, respectively; MS fragmentation pattern[M^+][Metastable]: 196[base peak], 181, 153, 132, 125, 110, 93, 77 and 65 m/z.

DISCUSSION

The fragmentation pattern, Figure 2 shows that the base peaks for both P-FMD and T-FMD are due to fragment (b), while that of N-FMD is due to fragment (c), because the substituent(s) on P-FMD and T-FMD are electron donating while that of N-FMD is electron withdrawing, as such the fragments of N-FMD is less stable than those of T-FMD and P-FMD. The D_{2h}-Point group symmetry in all the compounds is shown by the presence of fragment (a) corresponding, respectively to 207, 208 and 253 m/z for P-FMD, N-FMD and T-FMD. All the compounds show the presence of C-N (1280 to 1350 cm^{-1}) and C =N (1620 to 1650 cm^{-1}) functional groups on their IR charts. The peaks around 1100 and 1300 cm^{-1} confirmed the presence of ether linkage in T-FMD and P-FMD, while these peaks are absent in N-FMD but rather the peaks at 1350 and 1550 cm^{-1} are characteristics for symmetric and anti symmetric NO_2 stretches. The NMR spectra of T-FMD (^{1}H and ^{13}C) are simpler than those of P-FMD and N-FMD (it has fewer peaks). The 4-Methoxy group on T-

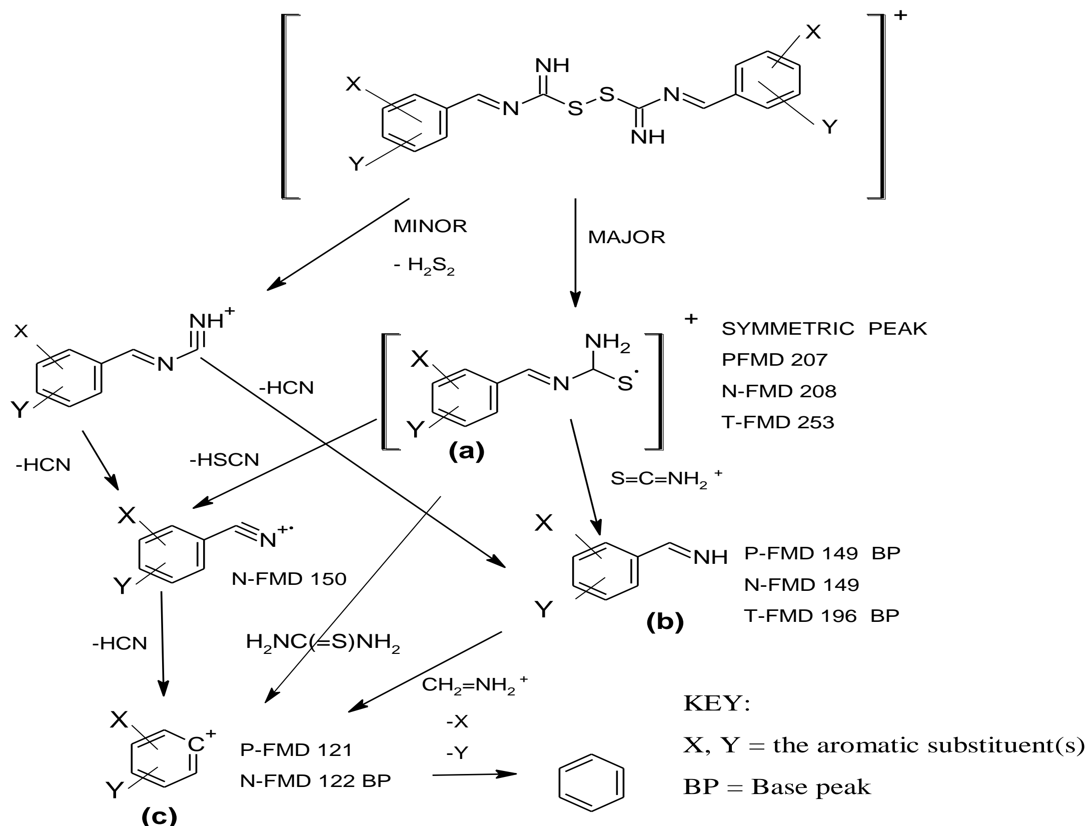

Figure 2. Fragmentation pattern of formamidine derivatives.

FMD provides another point of symmetry other than the disulphide bond which is common to the three compounds. The ^{13}C nmr spectra of all the compounds have a constant overlapping peaks at 190 ppm. These two peaks are attributed to the two imine carbons. The substituent(s) on the aromatic nucleus however helped at differentiating the various carbons as shown in Figure 1.

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Full Length Research Paper

Metabolic changes of glutathione in human T and B lymphocytes induced by organo-aluminum complex

Haroon Khan[1]*, Muhammad Farid Khan[1], Barkat Ali Khan[2], Naseem Ullah[1], Muhammad Mukhtiar[1] and Arshad Farid[3]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gomal University D.I. Khan Pakistan.
[2]Department 0f Pharmaceutics, Faculty of Pharmacy, Gomal University D.I. Khan Pakistan.
[3]Gomal Center of Biochemistry and Biotechnology, Gomal university D.I. khan Pakistan

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Even though aluminium is not considered to be a heavy metal like lead, silver, arsenic and cadmium, it can be toxic when taken in excessive amounts and even in small amounts if deposited in the brain. Glutathione, a major antioxidant in the cells, so its depletion weakens the tissue resistance to oxidant. Glutathione is the sulfhydryl (-SH) antioxidant, antitoxin and enzyme cofactor which plays an important role in aluminum detoxification. The present study was designed to investigate the extent of changes in glutathione level by inorganic and organic alumni metal. Biocordination of aluminum acetylacetonate and aluminum sulphate with glutathione in T-cells and b-cells of lymphocytes have been described using Ellman's method. The decline of glutathione level is due to increased aluminum concentration and time of incubation. The decline of glutathione level was consistent with increasing pH, while at physiological temperature, the drop was more significant. Our study indicates that changes in glutathione level produced by aluminium could be due to conjugate (Al-$(SG)_3$) formation. This change in glutathione level endowed with information regarding mechanism of toxicity of aluminium inorganic and organic complexes. This study is important for the design of rational antidote for the prevention of aluminium toxicity.

Key words: Glutathione (GSH), aluminium sulphate $Al_2(SO_4)_3$, aluminium acetylacetonate ($Al(acac)_3$) , T-cells, B-cells.

INTRODUCTION

Motivations for controlling heavy metal concentrations in gas streams are diverse. Some of them are dangerous to health or to the environment (for example, mercury, cadmium, lead, chromium), some may cause corrosion (for example, zinc, lead), some are harmful in other ways (for example, arsenic may pollute catalysts) (Michael, 2011). Some of these elements are actually necessary for humans in minute amounts (cobalt, copper, chromium, manganese, nickel) while others are carcinogenic or toxic, affecting among others, the central nervous system (manganese, mercury, lead, arsenic), the kidneys or liver (mercury, lead, cadmium, copper) or skin, bones, or teeth (nickel, cadmium, copper, chromium) and medical usage, heavy metals are loosely defined (Ron and Kilpinen, 2001) and include all toxic metals irrespective of their atomic weight: "heavy metal poisoning"

*Corresponding author. E-mail: haroonscholar@gmail.com. Tel: 0092-322-9830144

can possibly include excessive amounts of iron, manganese, aluminium, mercury, cadmium. All metals can cause disease through excess exposure. In addition, essential metals can affect the human body in the case of deficiency or imbalance. Until now, no biological function has been attributed to aluminium metal, and more importantly, aluminum accumulation in tissues and organs, results in their dysfunction and toxicity (Proudfoot, 2009; Verstraeten et al., 2008). No known physiologic need exists for aluminum; however, because of its atomic size and electric charge (0.051 nm and 3^+, respectively), it is sometimes a competitive inhibitor of several essential elements of similar characteristics, such as magnesium (0.066 nm, 2^+), calcium (0.099 nm, 2^+), and iron (0.064 nm, 3^+).

At physiological pH, aluminum forms a barely soluble compound $Al(OH)_3$ that can be easily dissolved by minor changes in the acidity of the media (Verstraeten et al., 2008). Aluminum causes an oxidative stress within brain tissue (Drago et al., 2008) since the elimination half-life of aluminum from the human brain is 7 years. This can result in cumulative damage via the element's interference with neurofilament axonal transport and neurofilament assembly. Some experts believe it plays a role in leading to the formation of Alzheimer-like neurofibrillary tangles.

Blaylock and Strunecka (2009) suggest that the heterogeneous symptoms of autism spectrum disorders have a connection with dysregulation of glutamatergic neurotransmission in the brain, along with enhancement of excitatory receptor function by proinflammatory immune cytokines as the underlying pathophysiological process. Animal studies in rats and case reports have implicated the use of oral aluminum-containing antacids during pregnancy as a possible cause for abnormal fetal neurologic development (Exley et al., 2009; Shuchang et al., 2008).

Glutathione (GSH) is a tripeptide that contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain. It is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Shuchang et al., 2003; Pastore et al., 2003). These free radicals are produce due to chronic exposer of aluminum metal (Priya and Bimla, 2006).

Many studies have been carried out in order to study the molecular mechanism responsible for the toxic effects of this metal ion on both humans and animals (Xiao et al., 2008). The results indicate that aluminum has been found inside cells in many illnesses (Vina et al., 2004; Xiao et al., 2008). Therefore, studying the binding of metal ion and its complexes by peptides and proteins is important for understanding many biological systems. It has been proposed that, in some cases, aluminum binds to certain natural carriers (Haroon et al., 2011). The interaction of aluminum with amino acids, peptides and proteins is a subject of current interest (Yves, 2000).

MATERIALS AND METHODS

L-Glutathione (GSH) was purchase from fluka chemical Co. All glutathione were prepared freshly daily with double distilled water, and all samples were flushed with high purity nitrogen or purified argon. Aluminium sulphate and aluminum acetyl acetonate were prepared by dissolving highly purified metallic aluminum powder of specific quantity in distilled water. More dilution were prepared by diluting these solutions with double distilled water. Roswell Park Memorial Institute (RPMI)-1640, fetal calf serum and ficol paque plus (Sigma) Ellman's reagent (DTNB) was obtained from sigma chemical Co. Other chemical reagents were of analytical grade. All measurements were taken on Schimadzu ultra violet (UV)/visible spectrophotometer. All glass wares were soaked in 10% HNO_3 for at least 24 h and then washed with double distilled water.

Preparation of stock solution

To make 0.9%-NaCl solution, 90 mg of pharmaceutical grade NaCl was added to distilled water quantity sufficient (q.s.) 100 ml. A 100 ml of 1 mM aluminum sulphate (molar weight (MW) = 342) solution was prepared by dissolving 34.2 mg of aluminium sulphate in 100 ml of distilled water, which was again gradually diluted to get 10 different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.00 mM) of aluminium sulphate. Aluminium acetyl acetonate [Al(acac)3], 1 mM solution contained 32.4 mg of $[Al(acac)_3]$ in D/W q.s. 100 ml, which was again gradually diluted to get 10 different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.00 mM) of $[Al(acac)_3]$. GSH (1 mM) solution was obtained through dissolving 15.375 mg of GSH in 50 ml of phosphate buffer (pH 7.6). A 19.8 mg of 5-5,Dithiobis 2-nitrobenzoic acid (DTNB) was added to phosphate buffer (pH 7.6) to get 50 ml of 1 mM solution of DTNB. Balanced salt solution was prepared by mixing 4-parts of fetal calf serum with 45 parts of RPMI-1640. Ficol paque plus was used without further purification.

Isolation of T-cells and B-cells

B and T lymphocytes were isolated according to the supplier's instructions of Ficoll-paque plus. Anticoagulant-treated blood (2 ml) collected from a healthy human volunteer was diluted with an equal volume of balanced salt solution (RPMI-1640 plus 10% fetal calf serum) (Flow laboratories) which was layered on the Ficoll-paque plus solution. This two-phase system was centrifuged at 400 × g for 30 to 40 min at 18 to 20°C. Differential migration during centrifugation resulted in the formation of layers containing plasma and different cell types. The bottom layer contained erythrocytes which sedimented completely through the Ficoll-paque plus. The layer immediately above the erythrocyte layer contains mostly granulocytes having density great enough to migrate through the Ficoll-paque plus layer. Because of their lower density, the lymphocytes accumulated at the interface between the plasma and the Ficoll-paque plus with other slowly sedimenting particles (platelets and monocytes). The lymphocytes are then recovered from the interface and subjected to short washing steps with RPMI-1640 to remove any Ficoll-paque plus and plasma. Platelet contamination was finally effectively removed with the 20% sucrose gradient layered over Ficoll-paque plus. The platelets remained at the top of the sucrose gradient and the lymphocytes sedimented

through the sucrose gradient to the top of the Ficoll-paque plus layer.

T-cells and B-cells controls

T-cells control was prepared through mixing isolated T-cells fraction and 0.9%-NaCl solution in 1:1 ratio without treating with metal solution. B-cells control was prepared through mixing isolated B-cells fraction and 0.9%-NaCl solution in 1:1 ratio without treating with metal solution.

Experimental design

Through centrifugation, plasma and cytosolic fraction were collected in separate sample tubes. The concentration dependent effect of aluminium sulphate and [Al(acac)$_3$] (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.00 mM) on GSH level was studied in T-cells and B-cells fractions each. The final concentrations of aluminium sulphate and [Al(acac)$_3$] (in each 10 samples of T-cells and 10 samples of B-cells fractions) were 50,100, 150, 200, 250, 300, 350, 400, 450 and 500 µM. Data for effect of aluminium sulphate and [Al(acac)$_3$] on GSH level at different time fixations (00, 30, 60 and 90 min) was also collected. The effect of aluminium sulphate and [Al(acac)$_3$] on GSH level in T-cells and B-cells was also studied at different pH (6.5, 7.6 and 8.5). The effect of different temperatures (25, 37 and 45°C), the time, and pH studies were carried out for change in GSH level in T-cells and B-cells by 10 different concentrations of aluminium sulphate and [Al(acac)$_3$] from 50 to 500 µM.

Experimental parameters

Assay of T-cells and B-cells fractions incubated with different aluminium sulphate and [Al(acac)$_3$] concentrations, at different time fixations, pH and temperature were made for estimation of effective toxicity marker compound, and glutathione in reduced form of GSH.

Determination of GSH concentration

The reduced glutathione level was determined by the method of (Ellman, 1959). Samples of the isolated T-cells and B-cells fractions (having different aluminium sulphate and [Al(acac)$_3$] concentrations) were mixed with each of the10 different concentrations of aluminium sulphate and [Al(acac)$_3$] in equal volumes. 0.2 ml from each of these mixtures was added to 2.3 ml of phosphate buffer (pH 7.6). Then 0.5 ml of reagent (DTNB) was added. The absorbance was determined at 412 nm against T-cells and B-cells control, respectively, where aluminium sulphate and [Al(acac)$_3$] had not been added. The concentration of GSH was expressed as µM.

Effect of aluminum sulphate on glutathione level adding into T-cell after separation

GSH level was determined in isolated T-cells after the addition of different aqueous solutions of aluminium sulphate, having the final concentrations of aluminium sulphate (50,100, 150, 200, 250, 300, 350, 400, 450 and 500 µM). The spectrophotometric analysis showed significant decrease in T-cells-GSH level (36.65 µM), with the initial aluminium sulphate concentration that is, 50 µM compared to the T-cells-control (0.445). The T-cells-GSH level was continuously decreasing in other samples which contained increasing aluminium sulphate concentrations. The maximum used aluminium sulphate concentration (500 µM) brought a drop in T-cells-GSH level up to (0.223), compared to the T-cells-control (0.445) as shown in Table 1 and Figure 1. Two aqueous solutions of 0.445 having final concentrations of 0.445 (50 and 500 µM) were incubated up to different time intervals (0, 30, 60 and 90 min) with isolated T-cells. By extending the time of incubation, the T-cells-GSH level was further decreased, with the maximum decrease in T-cells-GSH level being with the maximum provided time as shown in Table 1 and Figure 2.

Effect of aluminium acetylacetonate on glutathione level adding into T-cell after separation

GSH level was determined in isolated T-cells after the addition of different aqueous solutions of [Al(acac)$_3$], having the final concentrations of aluminium acetylacetonate (50, 100, 150, 200, 250, 300, 350, 400, 450 and 50 µM). The spectrophotometric analysis showed significant decrease in T-cells-GSH level (38.86 µM) with the initial [Al(acac)$_3$] concentration that is, 50 µM compared to the T-cells-control (0.458). The T-cells-GSH level was continuously decreasing in other samples which contained increasing [Al(acac)$_3$] concentrations. The maximum used [Al(acac)$_3$] concentration (500 µM) brought a drop in T-cells-GSH level up to (0.173) compared to the T-cells-control (0.458) as shown in Table 2 and Figure 3. Two aqueous solutions of [Al(acac)$_3$] having final concentrations of [Al(acac)$_3$] (50 and 500 µM) were incubated up to different time intervals (0, 30, 60 and 90 min) with isolated T-cells. By extending the time of incubation, the T-cells-GSH level was further decreasing, with the maximum decrease in T-cells-GSH level being with the maximum provided time as shown in Table 2 and Figure 4.

Effect of aluminum sulphate on glutathione level adding into B-cell after separation

GSH level was determined in isolated B-cells after the addition of different aqueous solutions of aluminium sulphate, having the final concentrations of aluminium sulphate (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µM). The spectrophotometric analysis showed significant decrease in B-cells-GSH level (39.27 µM) with the initial aluminium sulphate concentration that is, 50 µM compared to the B-cells-control (0.391). The B-cells-GSH level was continuously decreasing in other samples which contained increasing aluminium sulphate concentrations.

Table 1. Effect of aluminum sulphate on glutathione level adding into T-cell after separation.

No.	Time			50 µM	100 µM	150 µM	200 µM	250 µM	300 µM	350 µM	400 µM	450 µM	500 µM	Control
				0.345	0.329	0.319	0.299	0.285	0.273	0.259	0.240	0.233	0.223	0.445
			6.5	0.340	0.323	0.311	0.293	0.285	0.270	0.261	0.243	0.234	0.224	0.445
		pH	7.6	0.264	0.251	0.237	0.222	0.210	0.196	0.184	0.170	0.158	0.152	0.445
1	Average ABS at 0 min		8.5	0.288	0.275	0.262	0.246	0.235	0.221	0.208	0.194	0.182	0.176	0.445
			25	0.345	0.329	0.319	0.299	0.285	0.273	0.259	0.240	0.233	0.223	0.445
		Temperature (°C)	37	0.323	0.311	0.297	0.282	0.270	0.256	0.244	0.230	0.218	0.212	0.445
			45	0.336	0.321	0.313	0.294	0.284	0.273	0.257	0.239	0.231	0.222	0.445
2	Average ABS at 30 min			0.287	0.274	0.261	0.245	0.233	0.220	0.207	0.193	0.181	0.175	0.445
3	Average ABS At 60 min			0.260	0.248	0.234	0.219	0.207	0.193	0.181	0.167	0.155	0.149	0.445
4	Average ABS at 90 min			0.240	0.227	0.214	0.199	0.187	0.173	0.160	0.147	0.135	0.128	0.445

Table 2. Effect of aluminium acetylacetonate on glutathione level adding into T-cell after separation.

No.	Time			100 µM	200 µM	300 µM	400 µM	500 µM	600 µM	700 µM	800 µM	900 µM	1000 µM	Control
				0.360	0.333	0.316	0.293	0.273	0.257	0.236	0.213	0.199	0.173	0.458
			6.5	0.355	0.327	0.308	0.287	0.273	0.254	0.237	0.216	0.168	0.153	0.458
		pH	7.6	0.278	0.255	0.234	0.216	0.198	0.180	0.160	0.143	0.123	0.101	0.458
1	Average ABS at 0 min		8.5	0.303	0.280	0.259	0.241	0.223	0.204	0.185	0.168	0.148	0.126	0.458
			25	0.360	0.333	0.316	0.293	0.273	0.257	0.236	0.213	0.199	0.173	0.458
		Temperature (°C)	37	0.338	0.315	0.294	0.276	0.258	0.240	0.220	0.203	0.183	0.161	0.458
			45	0.351	0.325	0.309	0.288	0.272	0.256	0.234	0.212	0.196	0.171	0.458
2	Average ABS at 30 min			0.355	0.327	0.308	0.287	0.273	0.254	0.237	0.216	0.199	0.155	0.458
3	Average ABS at 60 min			0.275	0.252	0.231	0.213	0.195	0.177	0.157	0.140	0.120	0.098	0.458
4	Average ABS at 90 min			0.255	0.232	0.211	0.193	0.175	0.157	0.137	0.120	0.100	0.078	0.458

concentrations. The maximum used aluminium sulphate concentration (500 µM) brought a drop in B-cells-GSH level up to (0.183) compared to the B-cells-control (0.391) as shown in Table 3 and Figure 5. Two aqueous solutions of aluminium sulphate having final concentrations of aluminium sulphate (50 and 500 µM) were incubated up to different time intervals (0, 30, 60 and 90 min) with isolated B-cells. By extending the time of incubation, the B-cells-GSH level was further decreased with the maximum decrease in B-cells-GSH level being with the maximum provided time as shown in Table 3 and Figure 6.

Effect of aluminum acetylacetonate on glutathione level adding into B-cell after separation

GSH level was determined in isolated B-cells after the addition of different aqueous solutions of [Al(acac)$_3$], having the final concentrations of [Al(acac)$_3$] (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µM). The spectrophotometric analysis showed significant decrease in B-cells-GSH level (39.84 µM) with the initial [Al(acac)$_3$] concentration that is, 50 µM compared to the B-cells-control (0.398). The B-cells-GSH level was continuously decreasing in other samples which contained increasing [Al(acac)$_3$] concentrations.

Table 3. Effect of aluminum sulphate on glutathione level adding into B-cell after separation.

No.	Time			100 µM	200 µM	300 µM	400 µM	500 µM	600 µM	700 µM	800 µM	900 µM	1000 µM	Control
				0.269	0.256	0.252	0.237	0.226	0.220	0.209	0.194	0.191	0.183	0.391
			6.5	0.265	0.250	0.244	0.231	0.226	0.217	0.210	0.197	0.191	0.184	0.391
		pH	7.6	0.188	0.178	0.170	0.160	0.151	0.143	0.134	0.124	0.115	0.112	0.391
1	Average ABS at 0 min		8.5	0.213	0.203	0.195	0.185	0.176	0.168	0.158	0.149	0.140	0.136	0.391
			25	0.269	0.256	0.252	0.237	0.226	0.220	0.209	0.194	0.191	0.183	0.391
		Temperature (°C)	37	0.248	0.238	0.230	0.220	0.211	0.203	0.193	0.184	0.175	0.172	0.391
			45	0.260	0.248	0.245	0.232	0.225	0.219	0.207	0.193	0.188	0.182	0.391
2	Average ABS at 30 min			0.267	0.256	0.244	0.232	0.223	0.212	0.201	0.189	0.176	0.164	0.391
3	Average ABS at 60 min			0.240	0.229	0.218	0.206	0.197	0.185	0.175	0.162	0.150	0.138	0.391
4	Average ABS at 90 min			0.220	0.209	0.198	0.186	0.177	0.165	0.155	0.142	0.130	0.118	0.391

Table 4. Effect of aluminum acetylacetonate on glutathione level adding into B-cell after separation.

No.	Time			100 µM	200 µM	300 µM	400 µM	500 µM	600 µM	700 µM	800 µM	900 µM	1000 µM	Control
				0.314	0.291	0.276	0.250	0.229	0.215	0.197	0.175	0.167	0.155	0.398
			6.5	0.310	0.285	0.268	0.244	0.229	0.212	0.199	0.179	0.137	0.145	0.398
		pH	7.6	0.233	0.213	0.194	0.173	0.154	0.138	0.122	0.106	0.091	0.083	0.398
1	Average ABS at 0 min		8.5	0.258	0.238	0.219	0.197	0.178	0.163	0.147	0.130	0.116	0.108	0.398
			25	0.314	0.291	0.276	0.250	0.229	0.215	0.197	0.175	0.167	0.155	0.398
		Temperature (°C)	37	0.293	0.273	0.254	0.233	0.214	0.198	0.182	0.166	0.151	0.143	0.398
			45	0.306	0.284	0.269	0.245	0.228	0.215	0.196	0.175	0.165	0.153	0.398
2	Average ABS at 30 min			0.256	0.237	0.217	0.196	0.177	0.162	0.145	0.129	0.115	0.106	0.398
3	Average ABS at 60 min			0.230	0.210	0.191	0.170	0.151	0.135	0.119	0.103	0.088	0.080	0.398
4	Average ABS at 90 min			0.210	0.190	0.171	0.150	0.131	0.115	0.099	0.082	0.068	0.060	0.398

The maximum used [Al(acac)$_3$] concentration (500 µM) brought a drop in B-cells-GSH level up to (0.155) compared to the B-cells-control (0.398) as shown in Table 4 and Figure 7. Two aqueous solutions of [Al(acac)$_3$] having final concentrations of [Al(acac)$_3$] (50 and 500 µM) were incubated up to different time intervals (0, 30, 60 and 90 min) with isolated B-cells. By extending the time of incubation, the B-cells-GSH level was further decreased with the maximum decrease in B-cells-GSH level being with the maximum provided time as shown in Table 4 and Figure 8

DISCUSSION

In the present studiey, an attempt has been made to determine the effect of aluminum sulphate and aluminum acetyl acetonate on the glutathione level in T-cell and B-cell of human blood by the use of influential parameters like concentration and time of incubation. We have found. that there was a depletion of glutathione level in T-cell and B-cell by increasing the concentration and with the passage of time. The study was conducted in terms of determination of absorbance of glutathionespectrophotometicallyinT-cellandB-cell

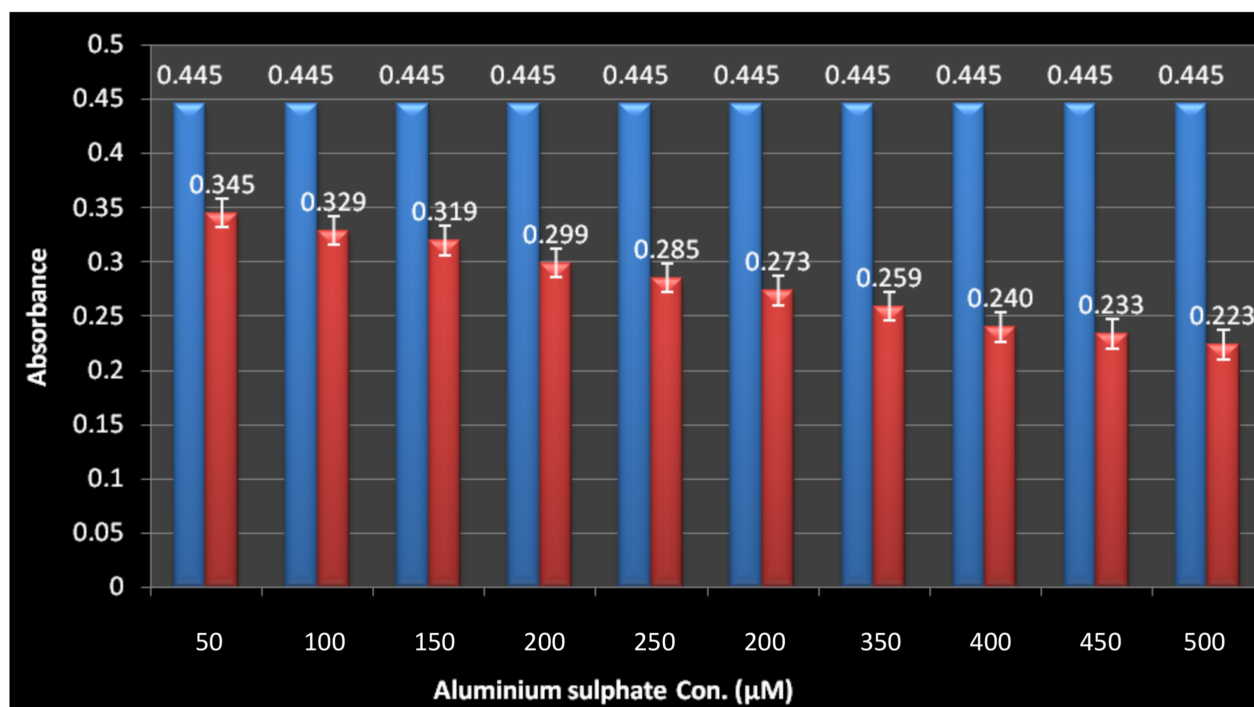


Figure 1. Effect of aluminium sulphate concentration on T-cells-GSH content. ■ T-cells Control (1 ml 0.9% NaCl + 1 ml of T-cells). ■ Aluminium sulphate (50 to 500 µM). Results are the mean ±SE of 3 experiments. Con = concentration.

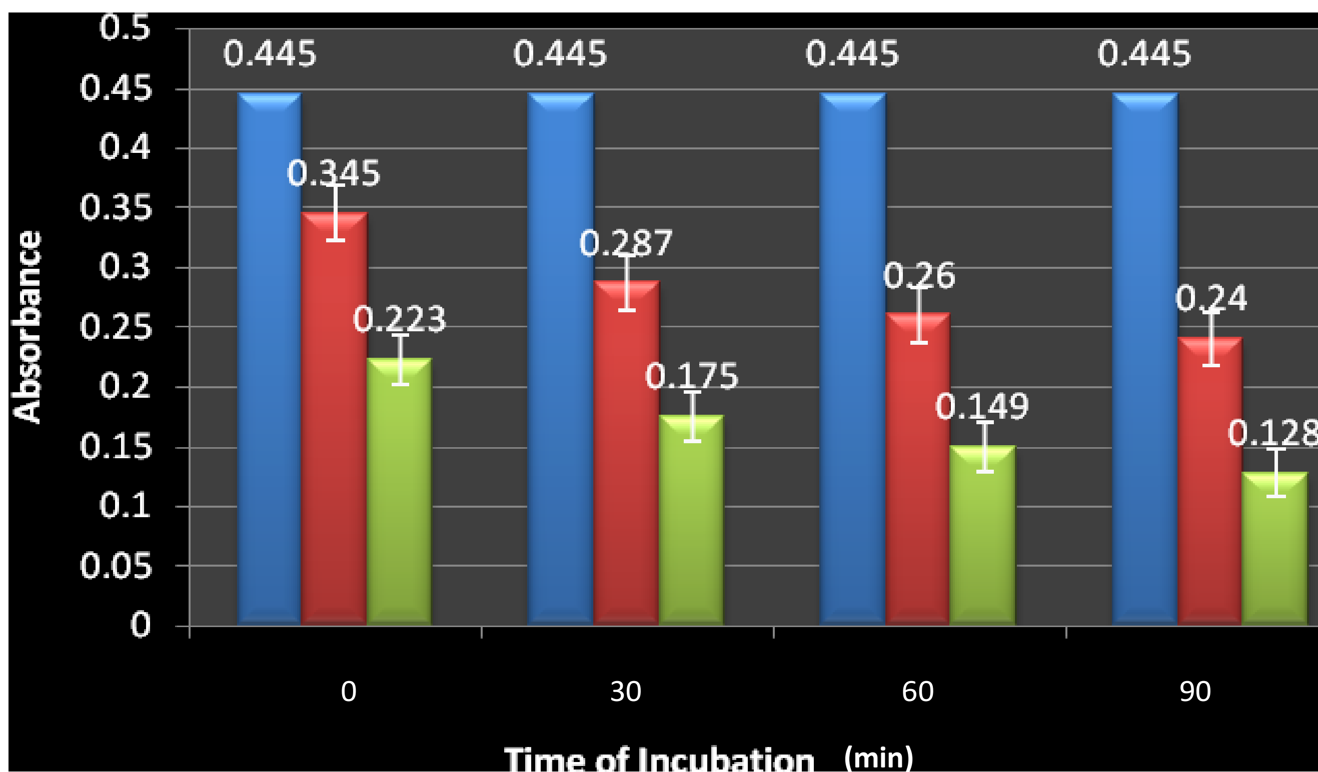


Figure 2. Effect of aluminium sulphate concentration on the T-cells-GSH content with time incubation period (0 to 90 min). ■ T-cells control (1 ml 0.9% NaCl + 1 ml of T-cells). ■ Aluminium sulphate (50 µM). ■ Aluminium sulphate (500 µM). Results are the mean ± SE of 3 experiments.

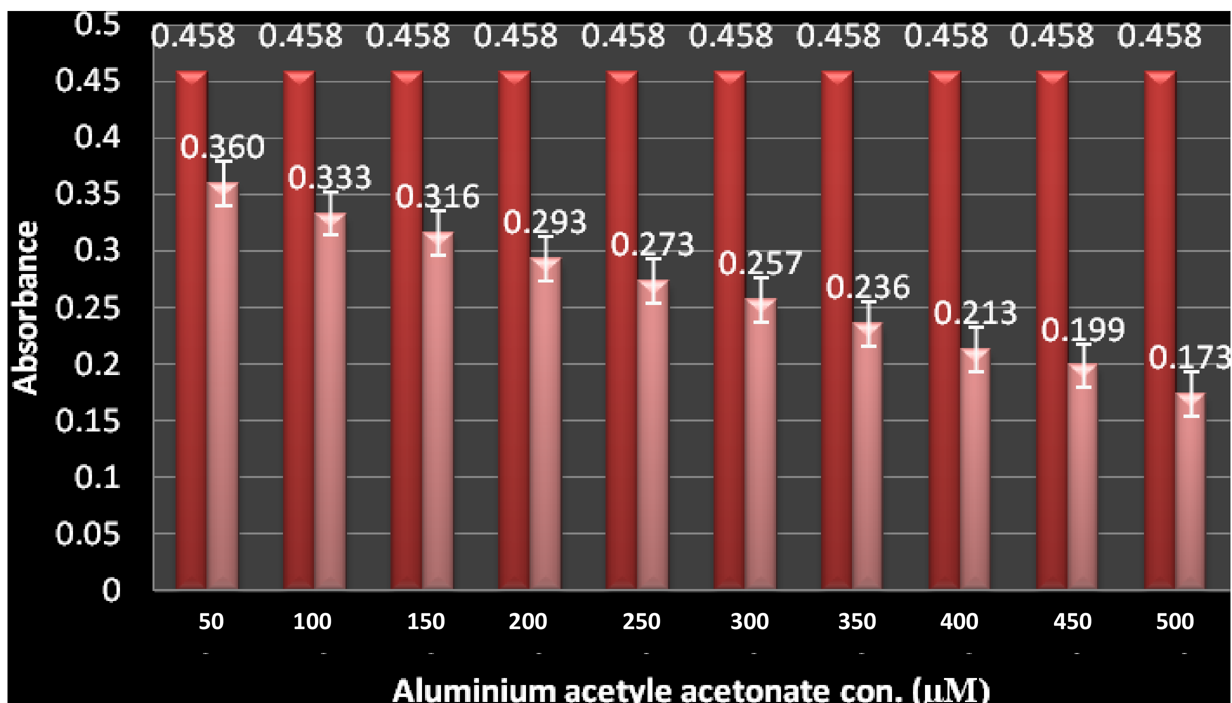


Figure 3. Effect of Aluminium acetylacetonate concentration on T.cells-GSH content. ■ T.cells control (1 ml 0.9% NaCl +1 ml of T-cells). ■ Aluminium acetylacetonate (50 to 500 µM). Results are the mean ± SE of 3 experiments. Con = concentration.

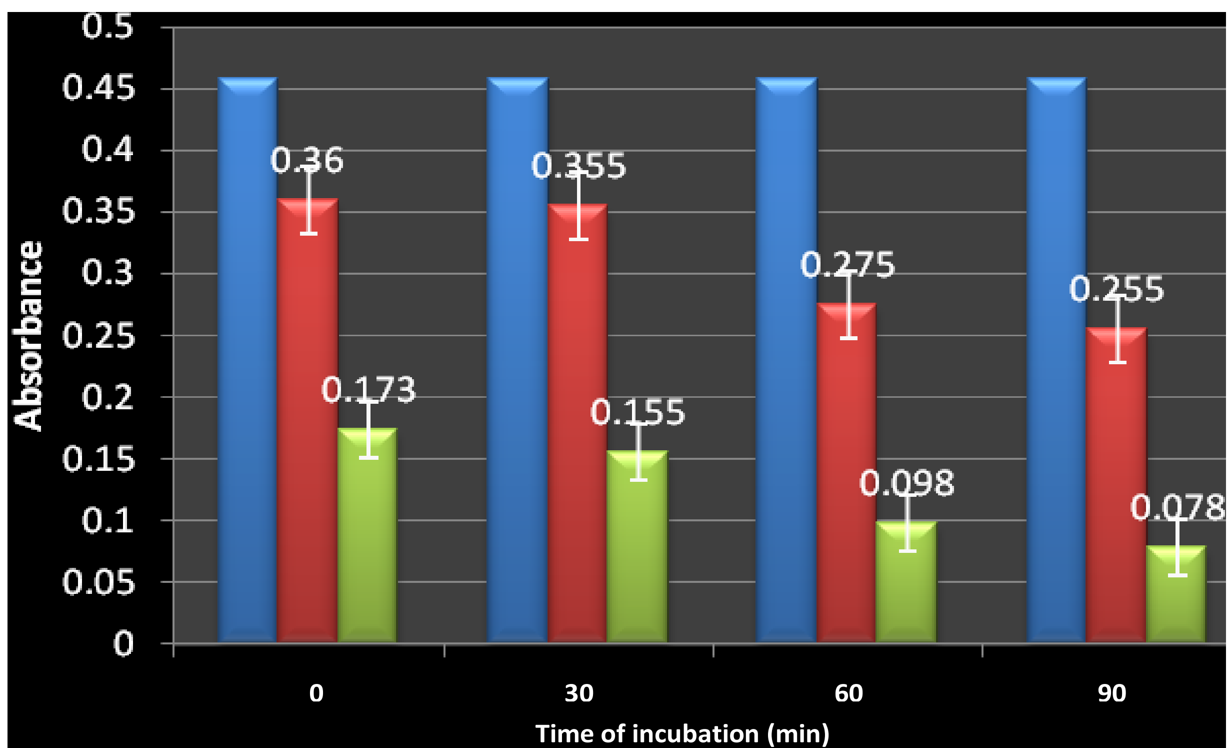


Figure 4. Effect of Aluminium acetylacetonate Concentration on the T.cells-GSH content with time incubation period (0 to 90 min). ■T-cells control (1 ml 0.9% NaCl +1 ml of T-cells). ■ Aluminium acetylacetonate (50 µM). ■ Aluminium acetylacetonate (500 µM). Results are the mean ± SE of 3 experiments.

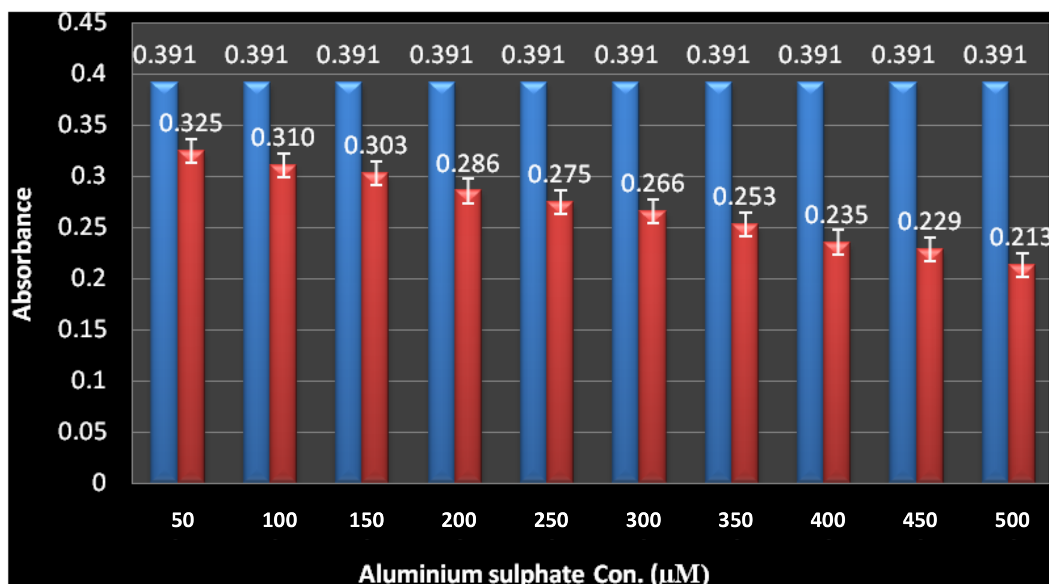


Figure 5. Effect of aluminium sulphate concentration on B.cells-GSH content. ■ B-cells control (1 ml 0.9% NaCl + 1 ml of B-cells). ■ Aluminium sulphate (50 to 500 µM). Results are the mean ± SE of 3 experiments. Con = concentration.

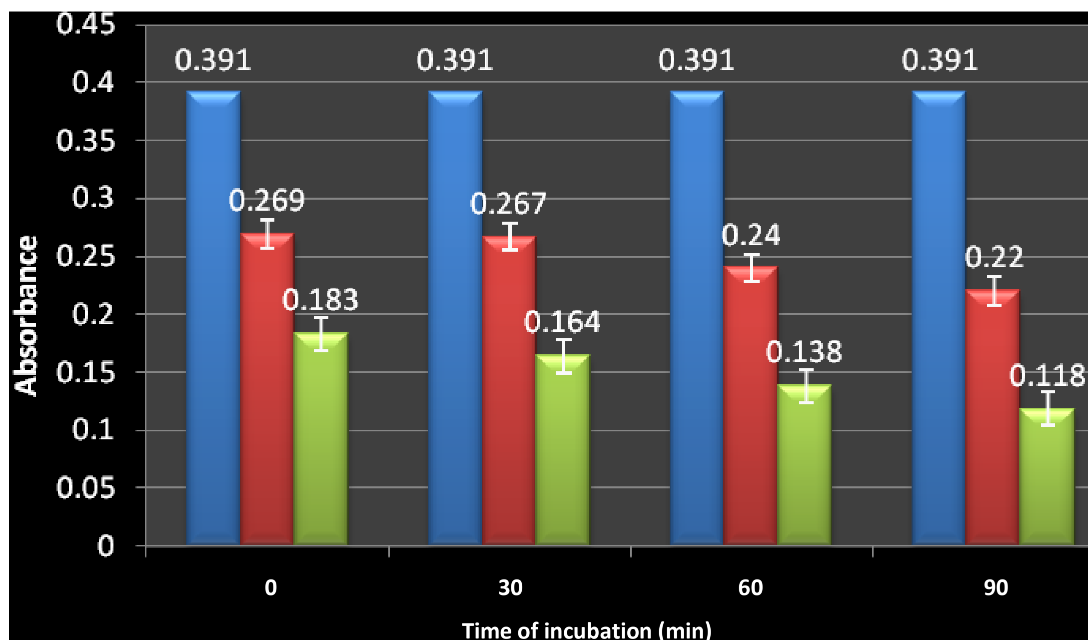


Figure 6. Effect of ATO Concentration on the B.cells -GSH content with time incubation period (0-90 min). ■ B-cells control (1 ml 0.9% NaCl + 1 ml of B-cells). ■ Aluminium sulphate (50 µM). ■ Aluminium sulphate (500 µM). Results are the mean ± SE of 3 experiments.

at ʎ max 412 nm by well-known Ellmans'method (Ellman, 1959). Our results confirm the finding report that aluminum has strong affinity with glutathione which in turn decrease the concentration of glutathione (Priya and Bimla, 2006).

Glutathione coordinates with Al (III) with an average efficiency, like simple monodentate amino acids. The carboxylate groups are effective binding sites for Al (III).

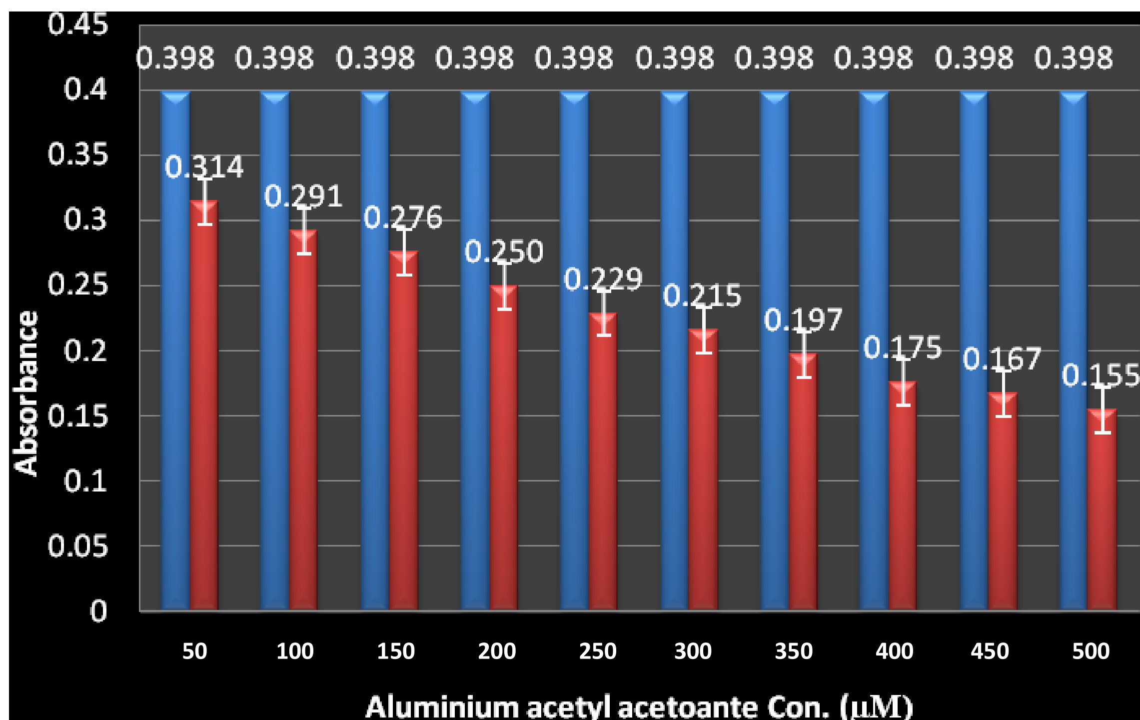


Figure 7. Effect of aluminium acetylacetonate concentration on B-cells-GSH content. ■ B-cells control (1 ml 0.9% NaCl + 1 ml of B-cells). ■ Aluminium acetylacetonate (50 to 500 µM). Results are the mean ± SE of 3 experiments. Con = concentration.

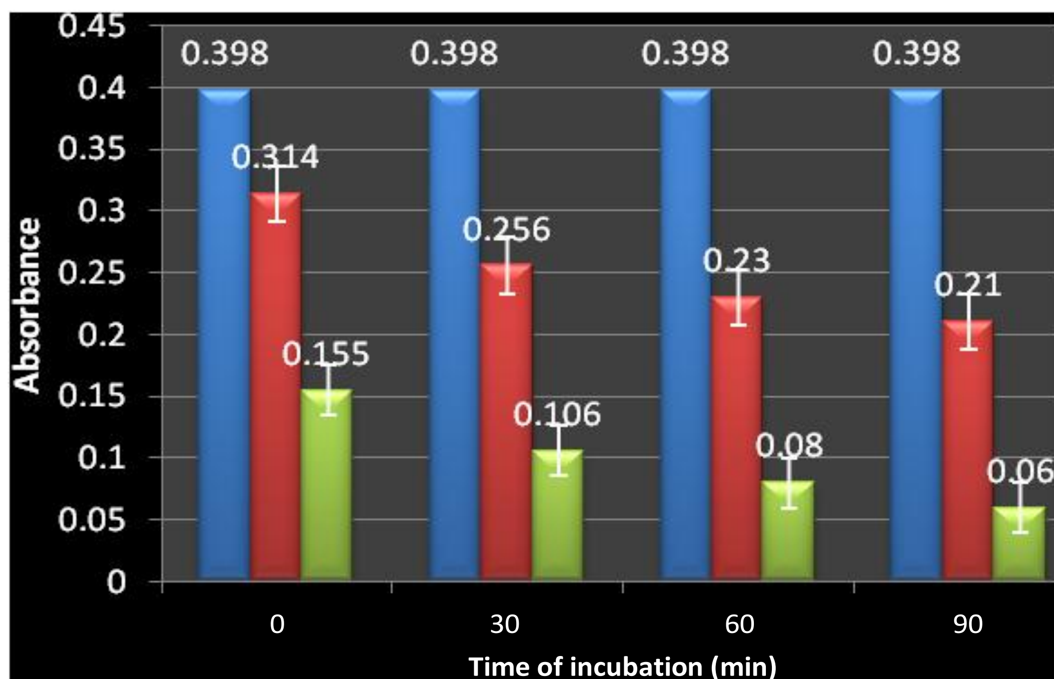


Figure 8. Effect of Aluminium acetylacetonate Concentration on the B.cells-GSH content with time incubation period (0 to 90 min). ■ B-cells control (1 ml 0.9% NaCl + 1 ml of B-cells). ■ Aluminium acetylacetonate (50 µM). ■ Aluminium acetylacetonate (500 µM). Results are the mean ± SE of 3 experiments.

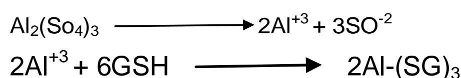
The possible binding sites are the negatively charged C-terminal Gly-COO- and Glu-COO groups. Unfortunately, this small peptide glutathione disulfide (GSSG) cannot prevent the precipitation of Al (III) complexes around the physiological pH range; it only keeps Al (III) ions in aqueous solution in an acidic pH range (Xiao et al., 2008). Our studies also confirm the findings report of (Haroon et al., 2011) that aluminium metal depleted the glutathione level in aqueous medium by increasing the influential parameters like concentration, time of incubation, pH and temperature. We also compare the studies with other findings and found that metals like silver, aluminum, lead, arsenic, mercury has high binding affinity for glutathione in aqueous as well as in blood components. The decline of glutathione level in aqueous and blood components is due to interaction of sulfhydryl group of glutathione and these metals (Haroon et al., 2011, 2012; Naseem et al., 2011).

Thus, the interaction of aluminium with reduced glutathione content of T-cells and B-cells *in vitro* as a model of *in vivo* reaction will establish further scientific data and will strengthen our knowledge about the toxicological profile of aluminium as well as the role of GSH in the protection of our body from their harmful effects. This also confirm the findings reported that adult rats are exposed to the aluminium dose 100 mg/kg bwt for 60 days, and registered a highly significant decrease in the level of glutathione as compared to control animal. According to our findings, the aluminium in the form of aluminum sulphate and aluminum acetyl acetonate induced the depletion of GSH content of T-cells and B-cells in a dose dependent manner. The results also show positive correlation between the exposure of glutathione content of T-cells and B-cells to the above given concentrations of aluminum sulphate and aluminum acetyl acetonate and the depletion of GSH content of T-cells and B-cells as the time passed from 0 to 90 min.

Studies by haroon et al. (2011) indicated that increase in the concentration of aluminum depleted the glutathione level in blood components. It also indicated that the decrease is more pronounced when time elapsed from 0 to 90 min. Furthermore, little information is available on the mechanism of action at the molecular level. Our experimental work proposes the molecular mechanism of action of aluminum sulphate and aluminum acetyl acetonate. Our research hypothesis was that aluminum sulphate and aluminum acetyl acetonate caused modulation in the status of GSH of T-cells and B-cells either to glutathione disulfide (GSSG) or formation of aluminum-glutathione (Al-SG) complex.

The exact mechanism of action of these compounds on GSH level in this study is not known. However, the proposed hypothetical mechanism of action of aluminum sulphate and aluminum acetyl acetonate on GSH level is the formation of $Al\text{-}(SG)_3$ complex.

The proposed reactions involve the coordination of Al^{+III} in aluminum sulphate and aluminum acetyl acetonate with the S-H group of GSH with the formation of $Al\text{-}(SG)_3$ complex as shown below



$$Al_2(So_4)_3 \longrightarrow 2Al^{+3} + 3SO^{-2}$$

$$2Al^{+3} + 6GSH \longrightarrow 2Al\text{-}(SG)_3$$

The importance of interaction of metalloelements including aluminium metal with GSH as a biomarker of detoxification may guide biochemical scientist to take account of metal salt/complexes and metal/ drug complexes for implementation into clinical settings

Conclusion

From these findings we concluded that when human B and T lymphates were exposes to aluminum sulphate and aluminum acetyl acetonate there was significant decrees in glutathione level present in T-cells and B-cells of lymphocytes of human blood. So the people exposed to aluminum may be more prone to Parkinsonism and alzimer as aluminum causes reduction in glutathione level which is natural antioxidant. It is further suppose that a targeted study may be conducted in future.

ABBREVATIONS

D/W; distilled water, **q.s;** quantity sufficient.

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