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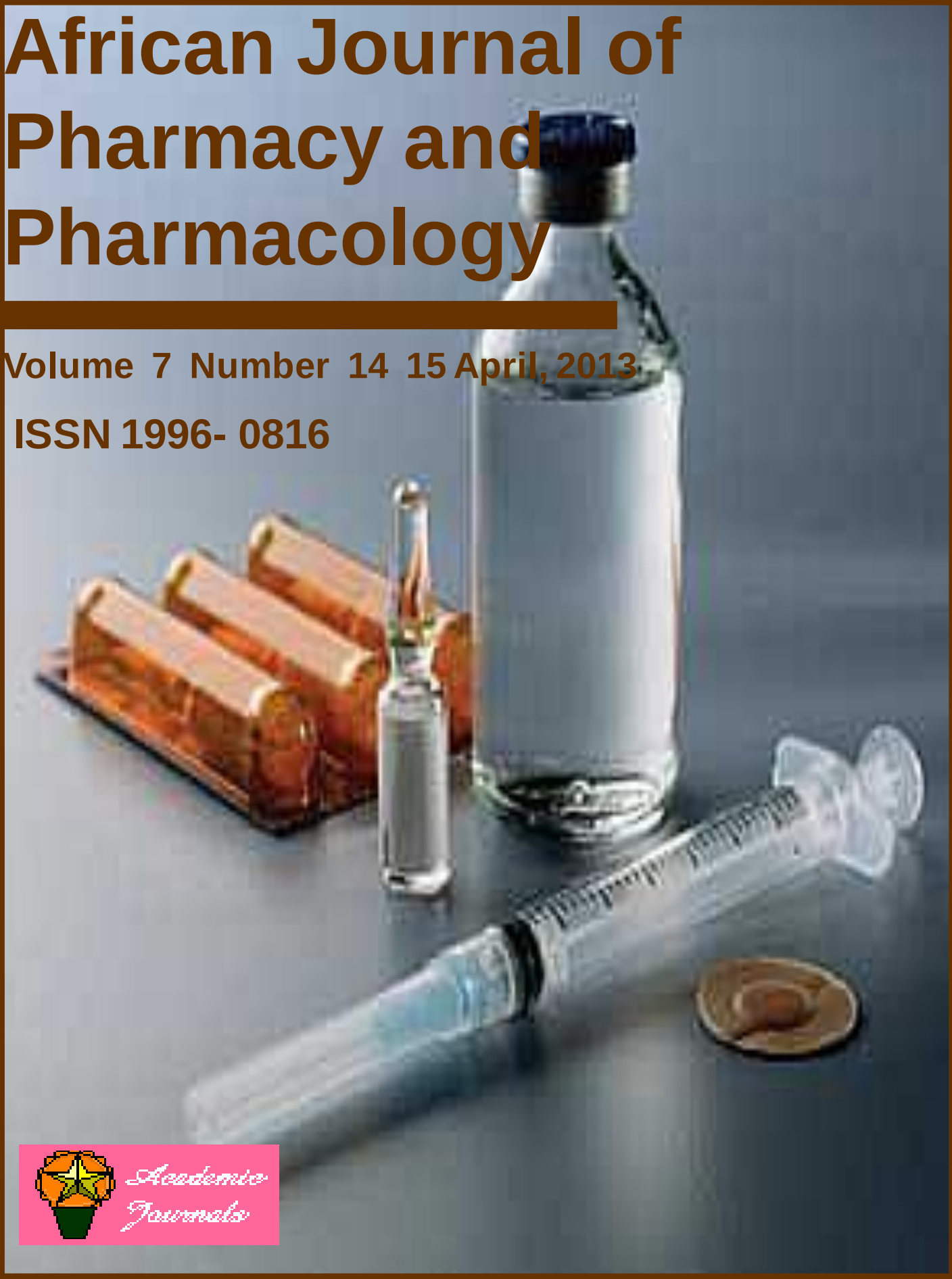

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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Evaluation of cytotoxic properties of *Curcuma longa* and *Tagetes erecta* on cancer cell line (Hep2)

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Cancer is the major public difficulty and one of the top causes of death in prosperous countries. Conventional plants are precious source of novel cytotoxic agents and play a critical role in health concern. Due to its wide range of biological and pharmacological effects and lack of toxicity, curcumin and lutein were selected for this study. Curcumin and lutein were isolated from rhizomes *of Curcuma longa* and petals of *Tagetes erecta*. The isolated pigments were quantified spectroscopically and separated by thin layer chromatography. The active components of the pigments were further purified and identified by high performance liquid chromatography. *In vitro* cytotoxic activity of both extracts against Hep2 cancer cell lines were evaluated. Furthermore, the activities of both pigments in different concentrations against Hep2 cancer cell line were compared. The test sample showing cell viability of more than 97% at 0.078 mg/ml were considered to be less active at minimum concentration. The maximum viability of Hep2 cell line were 3.27% (curcumin) and 8.88% (lutein), respectively, which are most suitable to perform cytotoxic studies. This method suggests that it is suitable for the rapid screening of plant materials and also can be performed without any special sample pretreatment.

Key words: Cytotoxic, curcumin, lutein, Hep2 cells.

INTRODUCTION

Cancer is a leading cause of death worldwide and had accounted for 7.9 million deaths (approximately 13% of all deaths) in 2007. Most drugs currently available for the treatment of cancer have limited potential, because they are highly toxic, inefficient in treating cancer, or highly expensive. Treatments without these disadvantages are needed. Hence, the identification and synthesis of novel, efficient and less toxic anticancer agents remains an important and challenging task for the cancer treatment. Use of plant extracts as medicine for cancer treatment is certainly the effective method and dozens of plant based products have been reported for cancer treatment progress. Since the plant based products have the natural multi-targeting ability as well as inexpensive and is safe as compared to synthetic agents (Preetha et al., 2008). Among them, plant based products such as curcumin and lutein occupied significant role against cancer, microbial infections and other inflammatory diseases. Due to its wide range of biological and pharmacological effects and lack of toxicity, curcumin and lutein were selected for this study.

Curcumin is a naturally occurring yellow pigment isolated from the rhizome of the perennial herb *Curcuma longa* which has been cultivated for centuries in several Asian countries. In general, the commercially available curcumin is a mixture of curcuminoids, containing

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diferuloylmethane, demethoxycurcumin and bisdemethoxycurcumin. Curcumin is known for its antioxidant, anti-inflammatory, anti-fatigue, antiparasitic, antiallergic, anti-microbial, anti-mutagenic and anticancer properties. It exhibits wide therapeutic potential due to the multi-targeting nature against variety of different cancers including leukemia, gastrointestinal cancers, genitourinary cancers, breast cancer, etc. Curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors. It also inhibits proliferation of cancer cells by arresting them in various phases of the cell cycle and by inducing apoptosis. It is obvious that curcumin's multitargeting ability may be the key to its therapeutic potential against cancer.

Carotenoids are a subclass of phytonutrients which are prominent in fruits and vegetables. Xanthophylls are a family of oxygenated carotenoids that contain hydroxyl or carbonyl groups that contribute to enhance their solubility and hence their distribution in animal tissues. Lutein is a xanthophyll that, together with zeaxanthin, has gathered increasing attention on the grounds of recent studies that show how an adequate intake of this product might help to prevent or ameliorate the effects of degenerative human diseases, such as age-related macular degeneration. Human plasma lutein has been inversely associated with cytochrome activity and human cancer. It is a known fact that humans do not synthesize lutein and depend entirely on dietary sources such as vegetables or supplement lutein pills. Marigold flower (*Tagetes Erecta* L) petals are a significant source of the xanthophyll, mainly lutein and have a much higher concentration of this pigment as compared to other plant materials. Marigold extracts have been commercialized internationally and are used as additives for poultry feed as they provide bright colors in egg yolks, skin and fatty tissues. It plays enormous biological role for them as chemopreventive agents which include cancer prevention, enhanced immune function, inhibition of the autooxidation of cellular lipids, etc. It is worth mentioning that we have little knowledge on the curcumin and lutein isolated from the Indian sub content plants. *C. longa* and *T. erecta* plants used for this study were collected from the local farmer place in Tamilnadu. The metabolically active compounds curcumin and lutein were isolated from *rhizomes of C. longa* and petals of *T. erecta.* The isolated active compounds were purified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Further, we have evaluated the cytotoxic activity of both the active compound against Hep2 cancer cell lines. Furthermore, we compared the activity of both pigments in cancer cell line with different concentrations.

MATERIALS AND METHODS

Plant and animal cells

The plant *C. longa and T. erecta* were collected from the premises of Tamilnadu Agricultural University (TNAU, Coimbatore) and Grow More Biotech, Hosur, India. The plant material was identified and authenticated by Assistant Professor Dharmaraj, Department of Botany, Ayya Nadar Janaki Ammal College, Sivakasi, Tamilnadu, India. The cell line used is human epithelioma cell line of larynx (Hep2) obtained from tissue culture section of Virology Department, King Institute of Preventive Medicine, Guindy, Tamilnadu, India.

Preparation of plant extract

The rhizomes of *C. longa* and petals of *T. erecta* were detached and dried for 7 days, and reduced to coarse powder using a hand blender. The powder (910 g) was subjected to continuous Soxhlet extraction using acetone (turmeric) and n-hexane (marigold flowers) (100%) at 50°C for 18 h to obtain the extract, which was concentrated in a rotary evaporator under reduced pressure. A fresh batch powder (500 g) was macerated in distilled water for 72 h, filtered, and freeze-dried.

Biophysical characterization and analysis

Spectrophotometric measurements were carried out using the Beckman DK-2 spectrophotometer inbuilt software. The detective wavelength was set at 420 and 445 nm, at which curcumin and lutein have their maximal spectrophotometric absorption. Similarly, saponification experiments were carried out as described earlier.

Thin layer and high performance liquid chromatography

Thin-layer chromatography was used to detect the individual curcuminoid in turmeric. Samples were dissolved in the appropriate organic solvent, applied to the silica gel G plates (Sigma Aldrich, India) and developed with petroleum ether, methanol and formic acid (3:4:0.5:0.1; v/v) developing solution. Further, the amount of curcumin and lutein was determined by HPLC (LC10 Shimadzu Corp., Tokyo, Japan) using C18 column. Chromatographic peaks of incubation samples were identified by spiking with corresponding authentic standards.

Cell culture, *in vitro* cytotoxic and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity tests define the upper limit of the extract concentration, which can be used in subsequent anticancer studies. Cells were harvested and separated to single cell suspension by gentle pipetting action and the viable cells were counted in a haemocytometer using trypan blue. Viable cell density was adjusted to 5,000 to 40,000 cells/100 µl. Hep2 cells were treated with different concentration of plant extract materials and observed morphological changes were observed under inverted microscope. After the addition of drug, cell death and cell viability was estimated. Furthermore, the cell survival was determined by using the MTT assay.

RESULTS AND DISCUSSION

The organic extraction of lutein from marigold flower and curcumin from turmeric is simple and less time consuming. Lutein extraction with saponification was performed, which does not allow the chlorophyll and other water soluble contaminants. The spectrophotometric

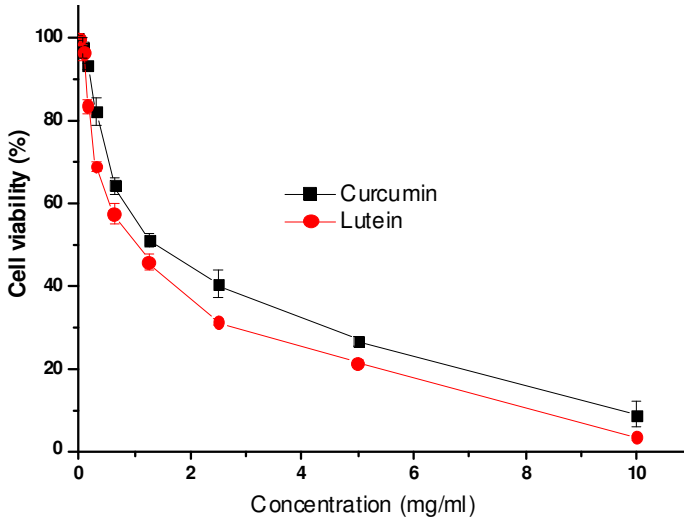

Figure 1. Cytotoxic effects of curcumin and lutein on Hep2 cells. Cells (3×105 cells/well), in 100 µl medium were grown in the presence of 0.4% DMSO (vehicle control) and various concentrations of curcumin and lutein. The numbers of viable cells were determined by MTT assay. The number of viable cells is expressed as a percentage of vehicle control. Mean ± standard deviation (SD) of 3 independent experiments.

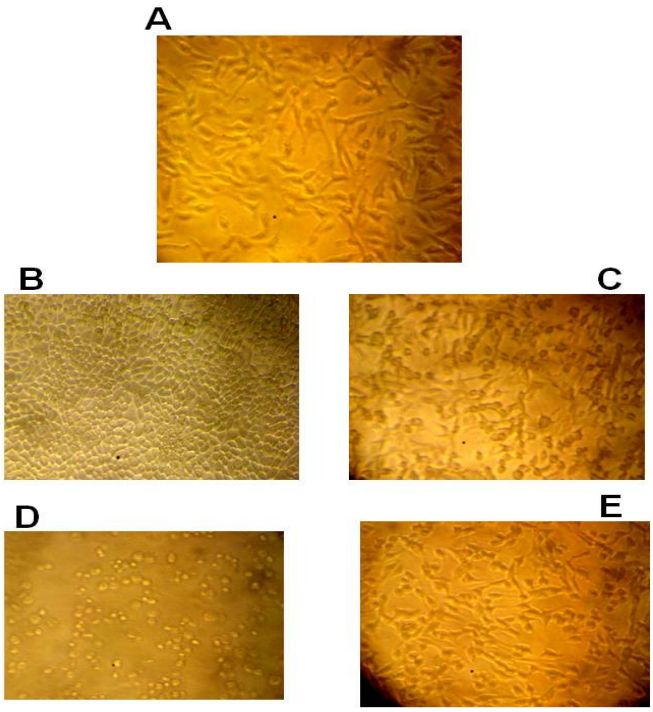

Figure 2. Microscopic images of cytotoxic effects of curcumin and lutein on Hep2 cells. Control (A) normal Hep2 cells without curcumin and lutein treatment; Test 1: Hep2 cells treated with curcumin (B) and lutein (C) with 0.3 mg/ml. Test 2: Hep2 cells treated with curcumin (D) and lutein (E) with 1.25 mg/ml.

method indicates the presence of several fractions from the curcumin and lutein isolated. Interestingly, strong peak was identified at 420 nm which is identical to those of curcumin standard solution (Ruby et al., 1995; Deshpande et al., 1997; Braga et al., 2003; Leal et al., 2003). We could observe the slight depression in the spectrum observed at ultraviolet (UV) region in comparison to lutein standard identified. It indicates that small amounts of polyphenols present in the hexane extract may interrupt during the spectrophotometric analysis. Furthermore, HPLC and TLC were used for the determination of individual curcuminoid in turmeric compounds as described earlier (Gupta et al., 1991). The isolated curcuminoids showed single spots on TLC plate and gave a single peak. The Rf value of curcumin extract is 0.74 and that of the standard is 0.77. Lutein content of the extract was determined by first saponifying the lutein esters in the extract which does not allow the chlorophyll and other water soluble contaminants except lutein with negligible amount of β-carotene. The separated spots of the saponified marigold color standard on TLC plate were identical with the spot of lutein standard in terms of Rf value and color. The Rf value of lutein extract after saponification is 0.45 (before saponification is 0.28) and the standard is of 0.44. Furthermore, the TLC purified samples were subjected to HPLC analysis. The HPLC analysis of curcumin and lutein showed the single peak and its retention time was 3.730 and 4.527 min, respectively. The retention time was comparable and exact to that of the standard curcumin and lutein peak. The solvents used for the elution of lutein are more suitable and reproducible with single peak (Thammanna et al., 2010).

The cytotoxicity study was carried out with the Hep2 cell lines at different concentrations to determine growth inhibition rate (Ju-Hyung et al., 2003). Dose response curves constructed between the range of 10 and 0.078 mg/ml for curcumin and lutein (Figure 1), express decreasing number of viable cells with increasing concentration of extract. The test sample showing cell viability of more than 97% at 0.078 mg/ml were considered to be less active at minimum concentration**.** The maximum viability of Hep2 cell line were 3.27% (Curcumin) and 8.88% (lutein), respectively, which are most suitable to perform cytotoxic studies. We therefore, can conclude that both plant extracts showed selective *in vitro* cytotoxicity against Hep2 cancer cell lines and both plants were found to be highly effective against various cancerous cells. The results were in accordance with previous research done in both plants by other authors. In the present study, both extracts proved to have effective cytotoxicity, but the growth inhibitory effect of lutein was maximum than curcumin. The anti proliferative effect strengthens with increase in the concentration of the extract. To verify curcumin-induced and lutein-induced cell toxicity, the changes in cell morphology were examined under an inverted microscope. High doses of curcumin and lutein were associated with increased cell apoptosis. Induction of apoptosis by curcumin appears to be dependent on the formation of reactive metabolites. Curcumin-induced apoptosis mainly involves the

mitochondria-mediated pathway in various cancer cells. Curcumin causes Hep2 cells to develop characteristic features of cell shrinking, rounding and partial detachment, thus demonstrating the lobulated appearance of apoptotic cells. We also examined the effects of different concentrations of curcumin and lutein on cell viability on Hep2 cells. After treatment, survival was inversely correlated with lutein concentration. When the cells were treated with 0.3 mg/ml of curcumin, 25% of cell death occurred (Figure 2). While decreasing the concentration of curcumin to 0.078 mg/ml and the viability of the cell peaks to 93.3%, respectively showing negligible amount of cell death and minimum lethal dose. We could observe that the 50% cell death could be seen at the concentration of 1.25 mg/ml where the viability was up to 50%. At 5 mg/ml concentration, the apoptosis rate was up to 75%. At 10 mg/ml, only 8.88% of viable were observed. Maximum cell death of 91.2% was observed at the concentration of 10 mg/ml. Very less amount of viable cells (8.8%) were detected at this concentration which shows the maximum inhibition concentration. Likewise when the cells were treated with lutein, the cell death was proportional to the concentration used same as curcumin. When the cells were treated with 0.3 mg/ml of lutein 25% of cell death occurred. Maximum viability of 96.72% was observed in the concentration of 0.078 mg/ml showing the lethal toxic rate. At the concentration of 1.25 mg/ml, the significance of viability was up to 46% leading to cell death of 44%. At 5 mg/ml concentration, the apoptosis rate was up to 78% (Figure 2). This finding will greatly benefit the clinical use of *T. erecta* in Indian medicine and suggests that lutein could be a potent anti-tumor drug candidate than curcumin. These findings validated the plant *T. erecta* as an antimicrobial herb.

ACKNOWLEDGEMENT

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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Pentoxifylline inhibits intimal hyperplasia and vascular smooth muscle cell proliferation in a rabbit carotid artery anastomosis model

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This study aimed to evaluate the inhibitory effect of pentoxifylline, a phosphodiesterase inhibitor on intimal hyperplasia (IH) and vascular smooth muscle cell proliferation in an anastomosis model of rabbit carotid artery (CA). Right CAs of 18 male New Zealand white rabbits were anastomosed under general anesthesia. After the surgical procedure, 18 rabbits were separated into three study groups, 6 in each. Group 1 (control group) did not receive any treatment. Group 2 and 3 were treated with 100 mg/kg/day subcutaneous pentoxifylline for 7 and 21 days, respectively. After 28 days, histopathological assessments and histomorphometric measurements were performed on the CA segments. In the histological sections, IH was less evident in the anastomosed vessel wall in pentoxifylline-treated groups than in Group 1. The mean luminal diameter (LD), luminal area (LA), intimal thickness (IT), and intima/media ratio (IMR) for Group 1 were 472.63 ± 13.28 µm, 301,973.33 ± 12,951.27 µm^2, 200,844.67 ± 8,375.38 µm, and 0.52 ± 0.01, respectively. The LD and LA were significantly higher, and the IT and IMR were significantly lower for Group 2 and 3 compared with Group 1 ($p < 0.05$). However, there was no significant difference between Groups 2 and 3 for these variables ($p > 0.05$). Subcutaneous pentoxifylline treatment, even for duration of only seven days decreases IH in arterial anastomosis sites in a rabbit CA anastomosis model.

Key words: Pentoxifylline, vascular graft, anastomosis, carotid arteries.

INTRODUCTİON

Reconstructive vascular surgery is a common intervention for the treatment of obstructive arterial disease that is less successful than expected due to the development of spontaneous thrombosis or restenosis after the surgery (Ducasse et al., 2003). While spontaneous thrombosis is responsible for the obstruction of the vessel in the early period, intimal hyperplasia (IH) leads to restenosis after surgery in the long-term.

Intimal hyperplasia (IH) is defined as the abnormal migration and proliferation of vascular smooth muscle cells with associated deposition of extracellular connective tissue matrix (Davies and Hagen, 1994; Subbotin, 2007). It develops in response to vessel wall injury, leading to luminal stenosis and occlusion (Davies and Hagen,

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1994; Bauters and Isner, 1997; Schwartz et al., 1992). Growth factors, hormonal factors, and mechanical factors play role in the process of IH (Bauters and Isner, 1997; Purcell et al., 1997). Prevention of IH after surgery is dependent on the pharmacologic suppression of subendothelial smooth muscle proliferation and synthesis of extracellular matrix (Sottiurai, 1990).

Pentoxifylline (PTX), a methylxanthine derivative, is a phosphodiesterase inhibitor with potent hemorheologic properties (Samlaska and Winfield 1994; Schönharting et al., 1988). It is used in the treatment of peripheral vascular disease, cerebrovascular disease, and a number of other conditions involving defective regional microcirculation (Ward and Clissold, 1987). It has anti-mitogenic effects and inhibits collagen synthesis; these effects are mediated predominantly through a cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) effector pathway (Chen et al., 1999).

Chen et al. (1999) showed that PTX inhibited proliferation and collagen synthesis in rat vascular smooth muscle cells under both basal and platelet-derived growth factor (PDGF) or transforming growth factor-beta (TGF-beta)-stimulated conditions. Hansen et al. (1999) found that PTX inhibited neointimal formation and induced constrictive vascular remodeling in a rat model of balloon injury by mechanisms involving decreased collagen type I production by vascular smooth muscle cells. In a recent study, it was shown that subcutaneous PTX treatment induced less proliferation within the vessel wall and increased lumen size after balloon angioplasty of the iliac artery of rabbits. It also has a positive effect on vascular remodeling (Busk et al., 2008).

On the basis of current evidence on the antimitogenic effects of PTX in animal models, we aimed to evaluate the effects of PTX on IH and vascular smooth muscle cell proliferation in an anastomosis model of the rabbit carotid artery (CA). We believe that the anastomosis model is superior to balloon angioplasty which has been used in previous studies, in that it is more similar to injuries that may occur during vascular surgery because it affects all three layers of the vessel wall.

MATERIALS AND METHODS

Experimental design and animals

This was a randomized, controlled experimental study. Eighteen male New Zealand white rabbits (2 to 3 kg) were used. All animals were kept in an air-conditioned environment in natural daylight at a room temperature of 20 ± 2°C. They were fed *ad libitum*. This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Dokuz Eylul University Local Ethics Committee for Animal Studies.

Surgical procedure

Before the surgery, a cannulae was inserted into the marginal ear vein for intravenous access. Surgical anesthesia was induced with 50 mg/kg of intramuscular ketamine and 5 mg/kg of xylazine (Rompun; Bayer, Munih, Germany). For infection prophylaxis, 50 mg/kg of intravenous cefazolin (Cefazol; Mustafa Nevzat, Istanbul, Turkey) was applied preoperatively. The right carotid artery (CA) was used for anastomosis and the left CA was left as control. The surgical area was prepared with 10% povidone iodine solution. A vertical incision was made on the right side of the neck and the right CA was explored. Following intravenous heparinization (100 IU/kg), the right CA was carefully dissected from the surrounding tissue. Bulldog-clamps were placed in the proximal and distal part of the CA, the artery was transected and anastomosed in an end-to-end fashion end-to-end using 8/0 polypropylene sutures. The clamps were then removed to re-establish blood flow (Figure 1). The anastomosis procedure was performed by the same investigator for all rabbits.

Experimental groups

After the surgical procedure, 18 rabbits were separated into three study groups, 6 in each. Group 1 (control group) did not receive any treatment. Group 2 and 3 were treated with 100 mg/kg/day subcutaneous PTX for 7 and 21 days, respectively. After 28 days, the right and left CAs of all animals were dissected free under general anesthesia and the arterial segments were sent to the Histopathology Laboratory for histological evaluation. Animals were euthanized with an intravenous overdose of pentobarbital sodium after 28 days. All of the rabbits lived for the entire study period without any neurological defects or wound infection.

Histopathological assessments

Specimens were fixed with 10% formaldehyde solution and embedded in paraffin. Serial cross-sections at 5 µm were obtained by cutting the paraffin blocks at the level of anastomosis with rotary microtome (Leica RM, 2135, Leica instruments, Nussloch, Germany). Afterwards, the specimens were stained with hematoxylin-eosin and Masson's trichrome. Sections of anastomozed and the corresponding contrlateral sides were evaluated under light microscope (Olympus BH-2, Tokyo, Japan). Photomicrographs were taken with a high-resolution video camera (JVC TK-890E, Japan).

Histomorphometric analysis

Photomicrographs were evaluated with digital image analysis software (UTSCSA; Image tool version 3.0, University of Texas, San Antonio, Texas, US). The thickness and area of the tunica media and intima, as well as the diameter and area of the vessel lumen, were stereologically measured for each vessel. The luminal diameter (LD) was calculated as the mean value of the three diameters. The luminal, intimal and medial areas were measured manually by encircling the area of lumen, internal and external elastic lamina. Afterwards, intimal area is calculated as the difference of the area of internal elastic lamina and luminal area (LA). The medial area is the substraction of the area of external elastic lamina from internal elastic lamina. Additionally, photomicrographs were converted into 3D images using Reconstruct 1.0.9.9 software (Excel Company; Brescia, Italy).

Statistical methods

The Statistical Package for the Social Sciences for Windows

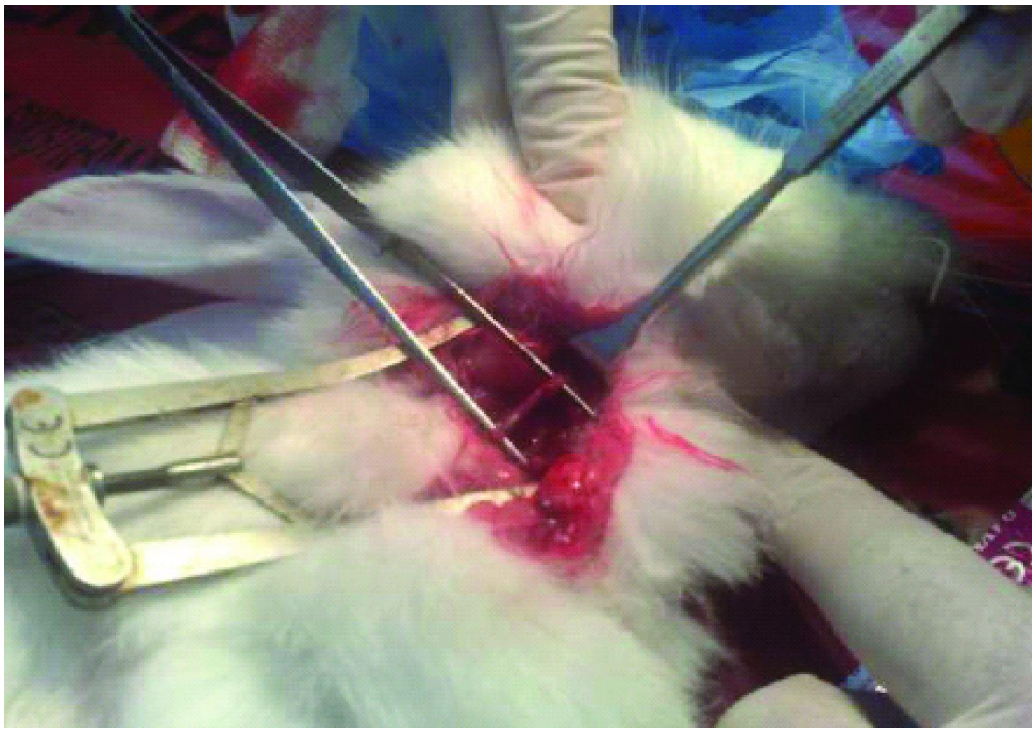

Figure 1. Exposure of the right carotid artery.

(version 11.0; Rel. 11.0.1. 2001, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to evaluate differences between experimental groups. For post-hoc multiple comparisons, the Bonferroni test was used. Statistical significance level was defined as $p < 0.05$.

RESULTS

Histopathological evaluation

In the sections of Group 1, the arterial lumen was almost entirely occluded, along with recanalizations in some sections. Other findings in these sections were IH due to smooth muscle cell proliferation, an increase in connective tissue, and intense fibrous tissue formation external to the tunica adventitia (Figure 2a). In contrast, the lumens of CAs of Group 2 were remarkably wider than the lumens of CAs of Group 1. In comparison with Group 1, the average intimal thickness (IT) was lesser and the tunica media was higher. However, recanalizations and adhesions, as well as areas of IH extending into the lumen were present in some sections (Figure 2b). In the sections of Group 3, the IT was less and the arterial lumen was wider than that of arteries from Groups 1 and 2 (Figure 2c).

In the histological sections of anastomosed right CAs, IH was less pronounced in the vessel walls of Group 2 and 3 than that of Group 1 (Figure 3). The structure of the intima and the lumen was normal in the sections of the contralateral left CAs in all three groups (Figures 2 and 3).

Three dimension (3D) reconstruction of ımages

In the 3D histological section images assembled using 3D Reconstruct for Windows 1.0.9.9, it was seen that IH was intense in Group 1, but lesser in Group 2 and 3. IT in Group 3 was lower than Group 2 (Figure 4). The vascular structures and lumen volume were normal in left CAs and were similar in 3D sections to those in all study groups (Figure 4).

Histomorphometric measurements

Luminal diameter

In Group 1, the LD was significantly lower in the right CA than the left (472.63 ± 13.28 µm versus 808.29 ± 11.27 µm, $p < 0.05$). In Groups 2 and 3, however, the LD of the right CA was significantly higher than that of Group 1 ($p < 0.05$). There was no significant difference between the LDs of Group 2 and 3 ($p = 0.865$) (Figure 5a).

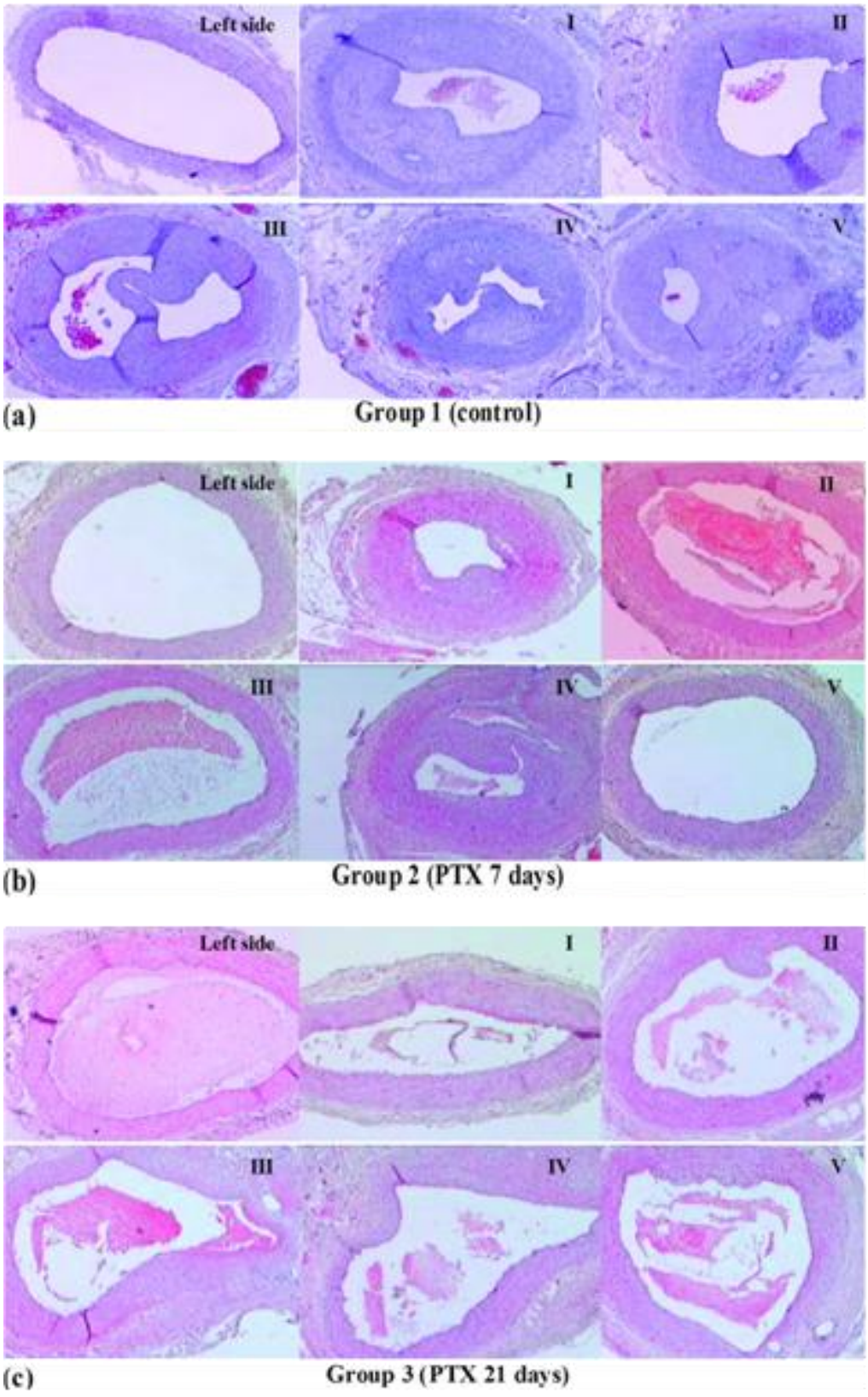

Figure 2. Histopathological assessment of the study groups.
*PTX: pentoxifylline.

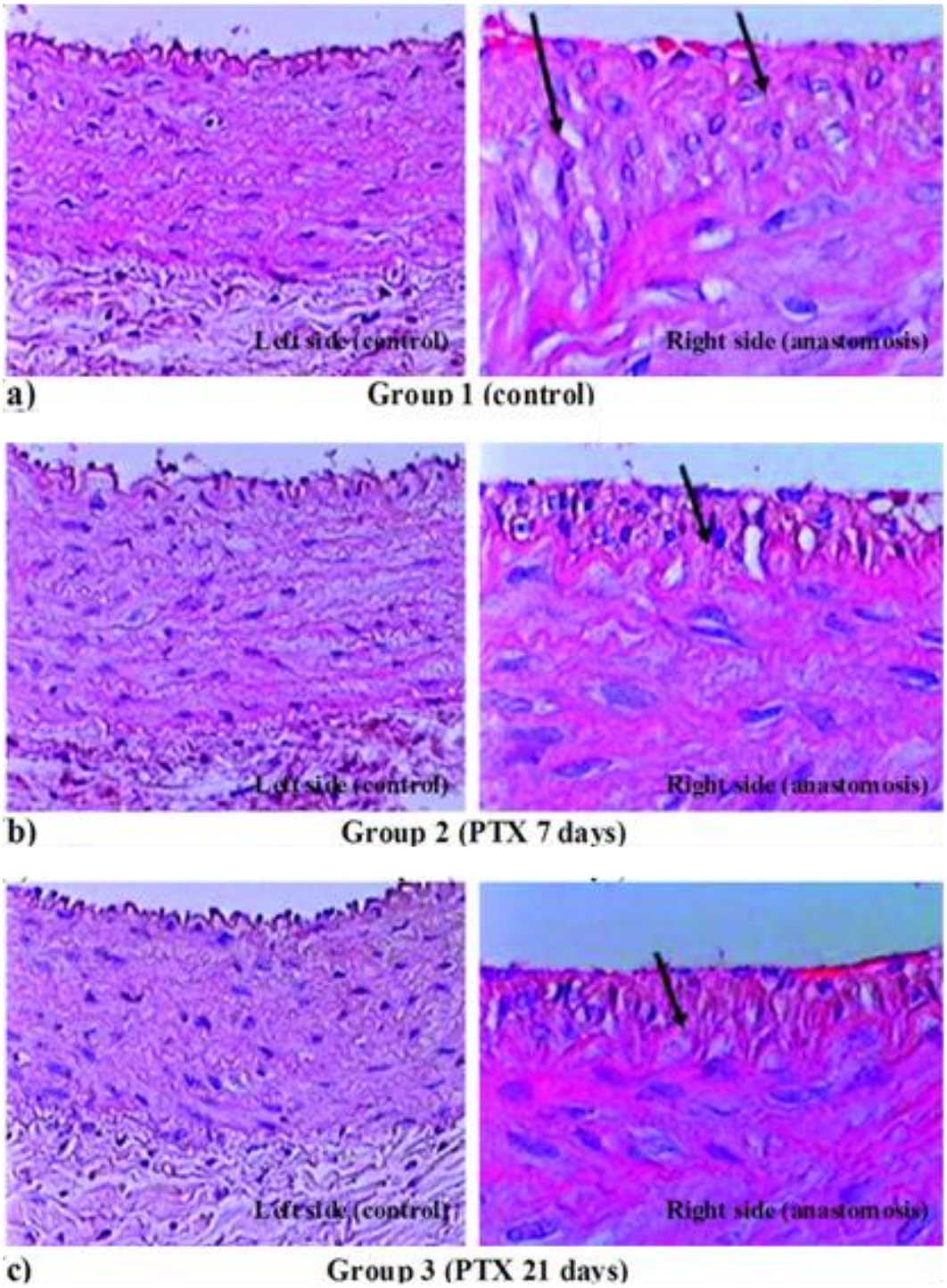

Figure 3. Comparison of left side (control) and right side (anastomosis) in histopathologic sections.

Luminal area

Measurements of serial cross-sections showed that the vascular LA of the right CA in Group 1 was lower than that of the contralateral side (301,973.33 ± 12,951.27 μm^2 versus 501,576.67 ± 13,104.00 μm^2), but this difference was not statistically significant ($p > 0.05$). In comparison with Group 1, the LA of right CA was significantly higher in Groups 2 and 3 ($p < 0.005$). Group 3 had a larger LA of anastomosed CAs than Group 2, but this difference was

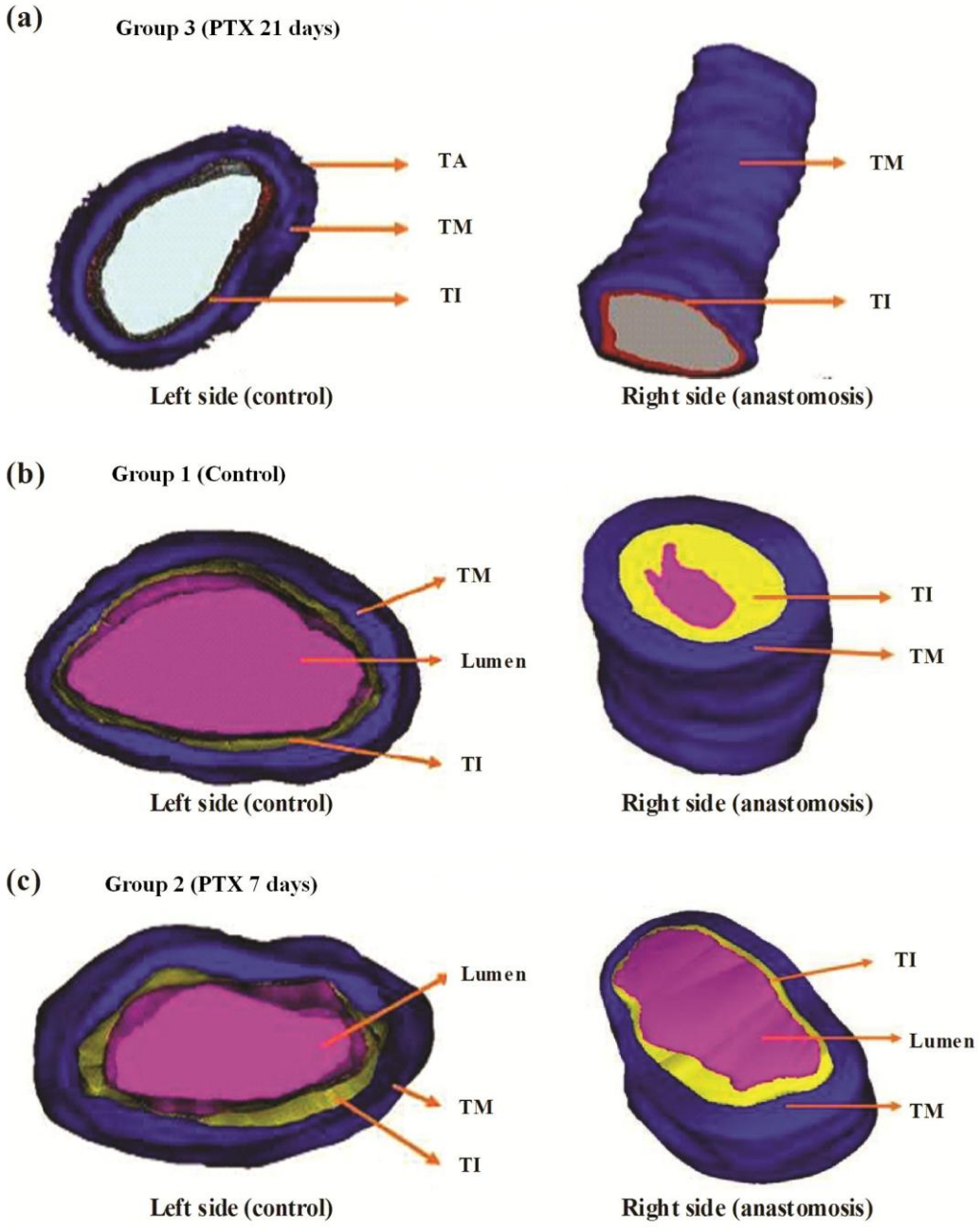

Figure 4. 3D reconstruction of the images of the vascular structures.
*TA: Tunica adventitia, TM: Tunica media, TI: Tunica intima. PTX: Pentoxifylline.

not significant (p = 1.000) (Figure 5a).

Intimal thickness

The mean IT was significantly higher in right CA than left in Group 1 (200,844.67 ± 8,375.38 µm versus 33,429.50 ± 817.50 µm, $p < 0.05$). The IT decreased significantly with PTX treatment ($p < 0.001$ for both Group 2 versus Group 1 and Group 3 versus Group 1). However, there was no significant difference between Groups 2 and 3 in terms of IT (p = 1.000) (Figure 5b).

Intima/media ratio

The ratio of the intima to media (IMR) in serial cross-sections was significantly higher in right than left for all

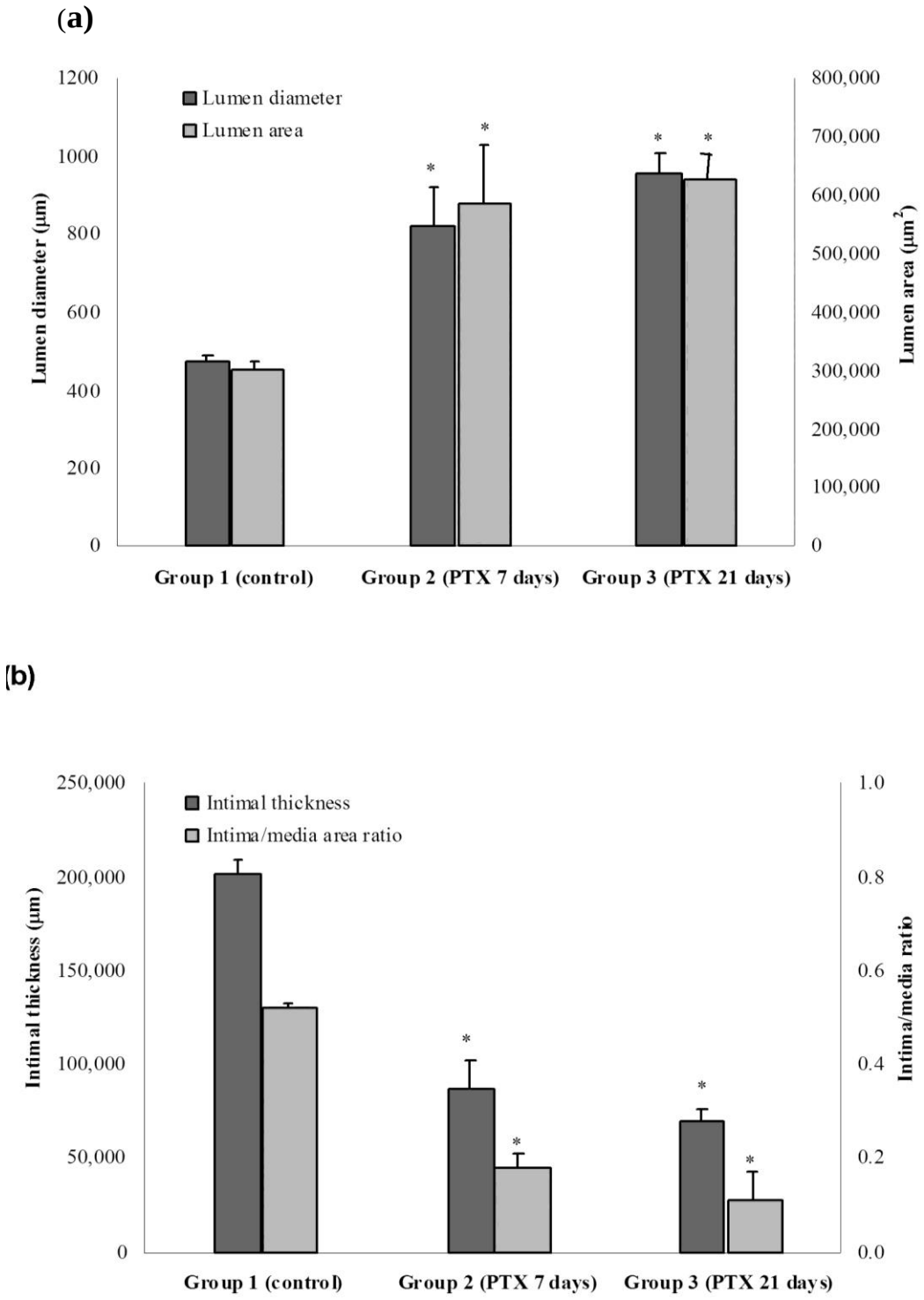

Figure 5. Measurements of luminal diameter-luminal area and intimal thickness-intimal/media area ratios in the study groups.
*PTX: pentoxifylline.

study groups ($p < 0.05$). The IMR of the anastomosed CA was 0.52 ± 0.01, 0.18 ± 0.03, 0.11 ± 0.06 for Groups 1, 2, and 3, respectively. The IMR was significantly lower in Groups 2 and 3 compared with Group 1 ($p < 0.05$ and $p <$

0.001, respectively) (Figure 5b). Although the mean IMR in group 2 was higher than that of group 3, there was no significant difference (p = 1). The left CA was not significantly different between study groups in terms of LD, LA, IT, and IMR (p > 0.05).

DİSCUSSİON

In this study, we evaluated the effects of PTX on IH and vascular smooth muscle cell proliferation in injured arteries by using an anastomosis model of rabbit CA. We found that subcutaneous PTX treatment for 7 or 21 days after CA anastomosis significantly increased the LD and LA; and decreased the IT and IMR in comparison with the control group that received no treatment. Although PTX treatment for 21 days resulted in more favorable results than treatment for 7 days, the difference between the 7-day and 21-day groups was not significant for any of the study variables. In spite of recent developments in vascular surgery, restenosis remains a primary postsurgical complication. Restenosis reduces the vascular lumen and ultimately induces thrombosis. IH and arterial remodeling have been identified as the underlying mechanisms of restenosis after vascular interventions (Bauters and Isner, 1997; Galt et al., 1993; Glagov, 1994). Therefore, IH is the major cause of failure after arterial reconstruction. The biology of IH is complex, and attempts at treatment have been disappointing (Subbotin, 2007; Zubilewicz et al., 2001). Although various agents have been studied for prevention of IH (O'Donohoe et al., 1991; Hamon et al., 1998; Takahashi et al., 1999; Takiguchi et al., 1995), restenosis continues to be a clinical problem after vascular surgery.

PTX increases the intracellular concentration of cAMP by inhibiting intracellular cAMP phosphodiesterase (Bienvenu et al., 1995; Mandell, 1995). Increased intracellular cAMP is thought to be responsible for most of the known effects of PTX, such as inhibition of vascular smooth muscle cell proliferation, reduction of blood viscosity, increased red blood cell deformability, decreased potential for platelet aggregation and thrombus formation, and inhibition of tumor necrosis factor (TNF)-alpha (Ward and Clissold, 1987; Bienvenu et al., 1995; Mandell, 1995; Strieter et al., 1988). PTX also suppresses inflammatory vascular damage and inhibits the effects of several cytokines (Mandell, 1995).

Previous studies on animal models using balloon angioplasty have shown that PTX reduces IH. Hansen et al. (1999) examined the effects of PTX in Sprague-Dawley rats treated with intraperitoneal PTX (75 mg/kg/day) or saline first dose being initiated 3 days before injury and continued until the 14th day after carotid balloon injury (total of 17 days). At 14th day of balloon injury, PTX significantly reduced the neointimal area, media area, IMR, and total vessel area. *In vitro*, PTX inhibits vascular smooth muscle cell production of collagen type I in a concentration-dependent manner without influencing vascular smooth muscle cell migration (Hansen et al., 1999).

Busk et al. (2008) recently investigated the effect of PTX on the vascular response to injury in a controlled study on rabbits that had undergone balloon angioplasty of the iliac artery, and treated with PTX (100 mg/kg/day) subcutaneously for 7 or 28 days after angioplasty or with placebo (saline) for 28 days after angioplasty. Although they did not compare the results of 7 and 28 day PTX treatment, they showed that angioplasty induced marked neoadventitial hyperplasia which was reduced by 20.5% (p = 0.01) in the PTX-treated group. PTX-treated rabbits had a 48.5% larger lumen area (p = 0.03) and a 28.1% larger area within the external elastic lamina (p = 0.04) at 28 days after injury. It has also been shown that PTX treatment leads to a more differentiated (less proliferation, more smoothelin-positive) intimal smooth muscle cell phenotype, reduced myofibroblast accumulation in the adventitia, and reduced collagen density in all three arterial layers (Busk et al., 2008).

In parallel to the findings of these previous studies, our findings showed that subcutaneous PTX treatment for 7 or 21 days after CA anastomosis significantly increased the LD and LA; and decreased the IT and IMR in comparison with the control group that received no treatment. Histological sections and 3D reconstruction images also showed less IH in PTX-treated groups compared with the control.

Previous studies employed various vascular models in rabbits for evaluating the biological response to vascular injury, as well as a diversity of therapies designed to modify intimal hyperplasia (O'Donohoe et al., 1991; More et al., 1994). To explore a more clinically relevant type of injury, we performed CA anastomosis on the rabbits as the basis of our vascular injury model. More et al. (1994) reported that, using the rabbit model of balloon angioplasty to the iliac arteries, intimal thickening characterized by myointimal hyperplasia was observed at day 7 and reached a maximum at 1 month. Thus we evaluated the CAs 28 days after the anastomosis procedure, since IH is known to reach a maximum at 28 days after arterial injury.

In our study, in contrast to the previous studies, we used a rabbit CA anastomosis model which used a balloon angioplasty model. In the balloon angioplasty model, we think only that the tunica intima is damaged due to shear stress. In the anastomosis model, on the other hand, all three layers of the vessel wall - the tunica intima, media, and adventitia are injured. In this respect, the anastomosis model better resembles injuries received during vascular surgery. Furthermore, this model provides an assessment of the effects of PTX on the injured media and adventitia layers in addition to the tunica intima.

The anastomosis model in our study induced IH in the CAs. In the histological sections from the anastomosed CAs of Group 1, occlusion of the arterial lumen was observed, along with recanalizations in some sections, IH due to smooth muscle cell proliferation, an increase in connective tissue, and intense fibrous tissue formation external to the tunica adventitia. In this study, we evaluated anastomosed right CAs in comparison with the contralateral left CAs (uninjured controls). We found that the intima and lumen structure, as well as the histo-morphometric measurements, were within normal limits in the sections from the left CA in all three groups, without any difference. The major limitation of this study was the difficulty of extending the animal study results to human clinical applications. Therefore, further human studies on this subject are required. On another point of view, different dosages of PTX with variable durations of applications should be compared. The results of our study are expected to provide an important basis for these further studies.

Conclusion

We found that subcutaneous PTX treatment, even for a duration of 7 days, decreased IH in a rabbit CA anastomosis model. Thus, although PTX treatment for 21 days induced more inhibition of IH, longer periods of treatment did not produce statistically significant differences in clinical outcomes.

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Full Length Research Paper

Resveratrol inhibits rat pulmonary fibroblast proliferation through modulation of TLR4/NF-κB pathway

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Resveratrol shows a powerful therapeutic effect in several cardiovascular diseases and cancer. Recent studies suggest a protective role of resveratrol in pulmonary fibrosis, but the underlying mechanism remains unknown. The aim of the present study is to investigate the anti-inflammatory effect of resveratrol on pulmonary fibrosis and reveal the role of toll like receptor 4 (TLR4) signaling pathway during the process. Pulmonary fibrosis (PF) model in rats was induced by endotracheal perfusion of bleomycin (BLM). Resveratrol treatment was given for three weeks. Blood samples and lung tissues were collected for further investigation. Results showed that the level of interleukin(IL)-1 and IL-6 in blood and lung tissue were both elevated in PF rats when compared with control rats (P<0.05). Resveratrol treatment significantly decreased IL-1 and IL-6 level (P<0.05). Real-time polymerase chain reaction (PCR) and western blot both showed that TLR4 expression in lung tissue from PF rats was up-regulated while resveratrol treatment significantly decreased TLR4 expression (P<0.05). Furthermore, primary rat pulmonary fibroblast was cultured. Lipopolysaccharide (LPS) induced cellular proliferation and increased TLR4 and NF-κB expression while NF-κB inhibitor BAY 11-7085 abolished the cell proliferation induced by LPS. Increased TLR4 and NF-κB expression induced by LPS incubation were both diminished by resveratrol (P<0.05). Cellular proliferation was also inhibited by resveratrol treatment. In conclusion, the anti-inflammatory effect of resveratrol may contribute its role in anti-proliferation of pulmonary fibroblast and PF therapy. TLR4/NF-κB signaling pathway is also involved in the process.

Key words: Pulmonary fibrosis, resveratrol, TLR4, NF-κB, inflammation.

INTRODUCTION

Pulmonary fibrosis (PF) is a severe clinical disease which could induce dyspnea, pulmonary hypertension and respiratory failure. Structural damage is the major characteristic of PF and usually happens prior to functional changes. Fibrotic tissues replace the normal lung parenchyma gradually (Noble et al., 2012), while proliferation of pulmonary fibroblast plays an important role during the process (Taniguchi et al., 2010).

The pathophysiology mechanism of PF has not been fully revealed so far. Most cases of PF may present as secondary outcome of other lung diseases, such as auto-immune disorders, viral infections or other microscopic injuries to the lung. Some cases can also be developed without any known cause, which was called idiopathic pulmonary fibrosis. It has been well recognized that inflammation was a common feature in various PF even in

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idiopathic cases (Bringardner et al., 2008), and toll like receptor 4 (TLR4) play a critical role in inflammatory response. Recently, several researches suggested that TLR4 signaling pathway is involved in PFprocess. It was reported that TLR4 activity was required in the resolution of pulmonary inflammation and fibrosis after acute and chronic lung injury (Yang et al., 2012). Treatment targeted on TLR4 signaling pathway was thought as an effective therapeutic strategy in PF treatment (John et al., 2010).

Resveratrol was widely used for protecting cardiovascular disease and cancer. Previous study has demonstrated that resveratrol, as a antioxidant, does not only decrease reactive oxygen species (ROS) but also show an anti-inflammatory effect (Bradamante et al., 2004). Recent research suggested that resveratrol treat-ment led to inhibit PF in bleomycin (BLM)-induced PF, while the underlying mechanism remains unknown (Akgedik et al., 2012). So the present study is designed to investigate whether the anti-inflammatory effect of resveratrol is involved in its therapeutic effect of PF. We also aim to explore the role of TLR4 signaling pathway during the process.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats weighing 150 to 180 g were obtained from Animal Center, Central South University (Changsha, China). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the experimental protocol was approved by the Medicine Animal Welfare Committee of Xiangya School of Medicine, Central South University.

Animal experiments

Twenty four Male SD rats were divided into three groups: control group, BLM perfusion group and BLM perfusion plus resveratrol (0.5 g/kg/day) group. There were 8 rats in each group. Endotracheal perfusion of BLM (5 mg/kg) was used to build PF model in rats. Resveratrol was given by intragastric administration continuously for 3 weeks. At the end of the experiment, the animals were anesthetized by sodium pentobarbital (30 mg/kg, intraperitoneally (ip)). Blood samples were collected by strength artery intubation. Plasma were prepared and frozen in -20°C for IL-1 and Il-6 enzyme-linked immunosorbent assay (ELISA) assay. After sacrificing the animals, the right, left lung lobes were dissected. The freshly isolated lung tissue samples were used for mRNA and protein expression analysis. Excised lungs were fixed in 4% paraformaldehyde for hematoxylin-eosin and immunohistochemistry staining.

ELISA and immunohistochemistry analysis of interleukin (IL)-1 and IL-6

Plasma concentration of IL-1 and IL-6 was determined by ELISA kits (R&D Systems Inc, Hong Kong, China), and the procedure according to the manufacturer's instructions.

Cell experiments

Pulmonary fibroblasts were prepared from the pulmonary lobe of male 10-week-old SD rats using explant method as described previously (Serlin et al., 2006). The cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum. The cells between passages 3 and 8 were used for the experiments. The cells were divided into 6 groups as follows: (1) Control: cells were treated with 21% O_2 for 48 h; (2) +lipopolysaccharide (LPS), cells were stimulated to proliferate by exposure to LPS (10 ng/ml) for 48 h; (3) +LPS plus low dose resveratrol (10 μM): cells were pre-treated with resveratrol for 1 h, and then subjected to LPS for 48 h; (4) +LPS plus medium dose resveratrol (50 μM): cells were pre-treated with resveratrol for 1 h, and then subjected to LPS for 48 h; (5) +LPS plus high dose resveratrol (100 μM): cells were pre-treated with resveratrol for 1 h, and then subjected to LPS for 48 h; (6) +LPS plus BAY 11-7085 (100 μM): cells were pre-treated with resveratrol for 1 h, and then subjected to LPS for 48 h.

Cell proliferation assays

Cell proliferation was measured according to the DNA synthesis by BrdU marking. For the BrdU incorporation assay, cells were counted and seeded into 96-well culture plates (6×10^3 cells per well). BrdU (10 μl/well, Roche, Mannhein, Germany) was added. Cells were fixed and stained after 4 h according to the manufacturer's instructions. Colorimetric analysis was performed with an ELISA plate reader (DTX880; Beckman, Miami, USA) at 450 nm.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from lung tissue and pulmonary fibroblasts, respectively by using TRIzol reagent (Invitrogen, China). 0.2 to 0.5 μg RNA was used for reverse transcription reaction using the PrimeScript reverse transcription reagent kit (TaKaRa, China). Quantitative analysis of the change in expression levels was performed using SYBR® Premix Ex Taq™ (TaKaRa, China) by the ABI 7300 system. PCR cycling conditions were an initial incubation at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 31 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the expression of mRNA. Primers for TLR4 were: forward5'-CCAGAGCCGTTGGTGTATC-3', reverse5'-GTGCCCTGTGAGGTCGTT-3'. Primers for IL-1 were: forward 5'-CCTGTGGCCTTGGGCCTCAA-3', reverse 5'-GGTGCTGATGTACCAGTTGGG-3'. Primers for IL-6 were: forward 5'- GAGAAAAGAGTTGTGCAATGGC-3', reverse 5' -ACTAGGTTTGCCGAGTAGACC-3'. Primers for GAPDH were: forward 5′-TGGCCTCCAAGGAGTAAGAAAC-3′, reverse 5′-GGCCTCTCTCTTGCTCTCAGTATC-3′. Data analysis was performed by comparative Ct method using the ABI software.

Isolation of nuclear extracts

Nuclear protein extracts were prepared according to the manufacturer's instructions. Pulmonary fibroblasts were collected, washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 400 μl total cell extract buffer A (10 mmol/L hydroxyethyl piperazineethanesulfonic (HEPES; pH 7.9), 10 mmol/L potassium chloride (KCl), 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L ethylene glycol tetraacetic acid (EGTA), 1 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulphonyl fluoride (PMSF)) for 15 min in ice. After vortexing, lysed cells were

centrifuged at 12,000 g for 3 min at 4°C, at which supernatant was removed by Pasteur pipette. 50 µl ice-cold cell extract buffer B (20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 25% (v/v) glycerine) was added into the nuclear pellets and vortexed at 4°C for 15 min. The samples were centrifuged at 12,000 g for 5 min at 4°C and the Aliquots of the nuclear protein extracts from supernatant were collected and stored at -70°C. Protein content of the nuclear extracts was determined using BCA reagent.

Electrophoretic mobility shift assay (EMSA)

NF-κB activity was performed by EMSA. Nuclear extracts containing 15 µg total protein were incubated with double-stranded NF-κB specific oligonucleotide probe end-labeled with [γ-32P] ATP using T4 polynucleotide kinase. The double-stranded DNA probe sequence is 5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5' (antisense). The labeled probe was purified through Sephadex G-25. DNA-protein binding reactions were incubated for 15 min on ice to allow complex formation. After 10 min of incubation at room temperature, the samples were subjected to electrophoresis on 4% non-denaturing polyacrylamide gel at 250 V in 0.5X Tris–borate–EDTA (TBE) running buffer for 2 h. After electrophoresis, the gel was then dried and the DNA-protein complexes were detected by autoradiography. After being dried, the gel was exposed to X-ray film at -70°C for 6 to 48 h.

Western blot analysis

Protein was extracted from lung tissue and pulmonary fibroblasts with radioimmunoprecipitation assay (RIPA) buffer (containing 0.1% PMSF), and equal amounts of protein from each sample (30 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with primary antibodies overnight at 4°C, and horseradish peroxidase (HRP)-coupled goat anti-mouse secondary antibody (1:2000, Santa Cruz, California, USA). The chemiluminescence signals were detected with the EasySee Western Blot Kit (Beijing TransGen Biotech, Beijing, China). The densitometric analysis was conducted with Image J 1.43 (National Institutes of Health). Primary antibody against TLR4 (ab22048 and ab13556, 1:1000 dilution) and NF-κB (Anti-NF-κB p65, acetyl K310, 1:1000 dilution) was purchased from Abcam (Hong Kong, China), and primary antibody against GAPDH (1:2000 dilution) was obtained from Santa Cruz (California, USA).

Statistical methods

The results were presented as means ± SEM (standard errors). Statistical analysis was performed by ANOVA followed by Newman-Student-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

Inflammatory response in BLM-induced rats

Plasma was collected from all rats, then inflammation factors concentration in plasma were determined using ELISA method. IL-1 concentration in plasma from BLM injected rats was significantly elevated while resveratrol treatment decreased IL-1 level (Figure 1A). Similar to IL-1, IL-6 concentration in plasma from BLM injected rats was also significantly elevated. But resveratrol treatment only slightly decreased its level without statistical differences (Figure 1B).

Resveratrol inhibited proliferation of pulmonary fibroblast induced by LPS

The direct effect of resveratrol on pulmonary fibroblast proliferation was first investigated. Cells were cultured for 48 h and cell proliferation was determined by BrdU marking. LPS (10 ng/ml) incubation stimulated a significant proliferation of pulmonary fibroblast while resveratrol pre-incubation inhibited the effect (Figure 2).

Resveratrol down-regulated TLR4 expression *in vivo* and *in vitro*

To observe the activation of TLR4 signaling, protein was extracted from lung tissues. Then TLR4 protein expression was determined using western blot. The protein expression of TLR4 in lung tissue from BLM injected rats was significantly increased while resveratrol treatment decreased TLR4 level (Figure 3).

We investigate the direct effect of LPS and resveratrol on TLR4 expression in cells. Pulmonary fibroblast was cultured with LPS with or without resveratrol for 24 h. RNA and protein were collected and detected by real-time PCR and western blot, respectively. LPS (10 ng/ml) incubation significantly increased TLR4 mRNA expression which was inhibited by resveratrol in dose-dependent way (Figure 4A). Western blot showed the similar changes of TLR4 protein expression. TLR4 expression was induced by LPS but significantly inhibited by resveratrol in dose-dependent way (Figure 4B).

Role of NF-κB in pulmonary fibroblast proliferation regulation

Furthermore, to investigate the role of NF-κB in proliferation regulation by LPS, BAY 11-7085, a NF-κB inhibitor was used. Pre-treatment of BAY 11-7085 significantly inhibited NF-κB expression induced by LPS (Figure 5A). And we found that pre-treatment of BAY 11-7085 cancelled the pulmonary fibroblast proliferation induced by LPS (Figure 5B), which suggest a critical role of NF-κB in pulmonary fibroblast proliferation regulation.

Resveratrol inhibited LPS-induced NF-κB activation in pulmonary fibroblast

We investigate the direct effect of LPS and resveratrol on NF-κB expression and activity in cells. Pulmonary fibroblast

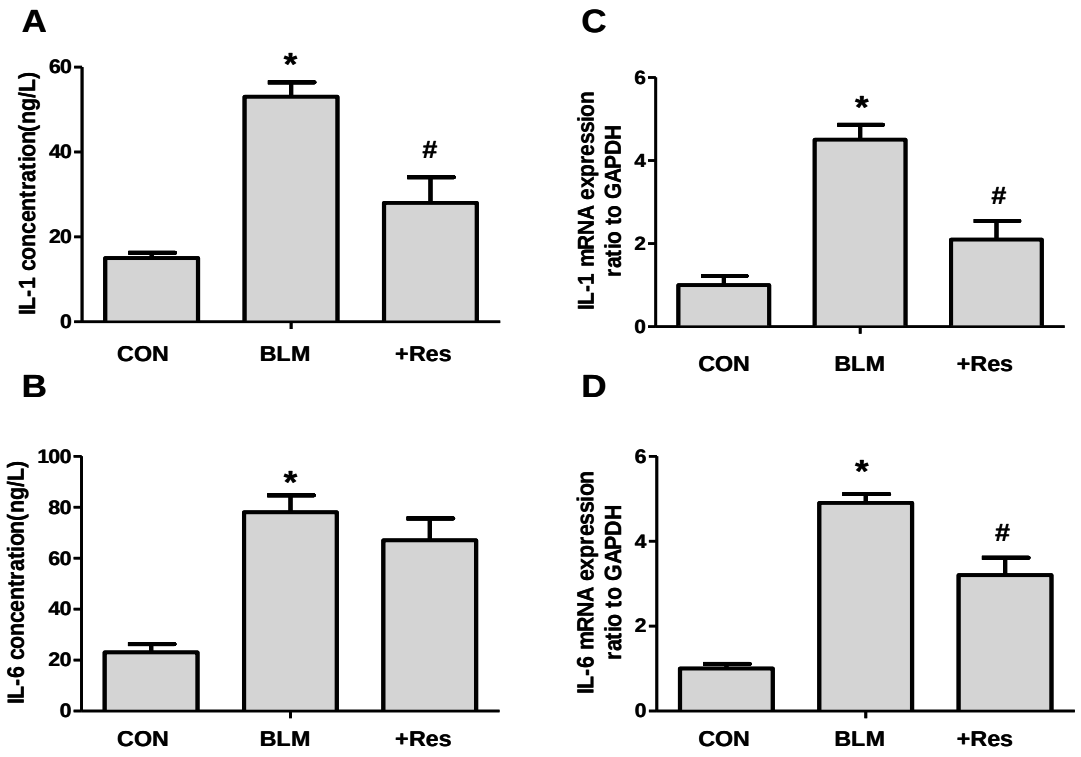

Figure 1. Plasma concentration and tissue expression of inflammatory factors. Inflammation factors concentration in plasma were determined using ELISA method. Tissue expression of inflammatory factors were determined by real-time PCR. (A) IL-1 concentration in plasma from BLM injected rats was significantly elevated (52.7±4.3 vs. 17.3±2.4 ng/L) while resveratrol treatment decreased IL-1 level (24.6±5.7 ng/L). (B) Similar to IL-1, IL-6 concentration in plasma from BLM injected rats was also significantly elevated (78.3±4.5 vs. 22.4±2.8 ng/L). But resveratrol treatment only slightly decreased its level without statistical differences (65.8±6.1 ng/L). (C) IL-1 mRNA expression in lung tissue from BLM injected rats was significantly increased, which was down-regulated by resveratrol treatment. (D) IL-6 mRNA expression in lung tissue from BLM injected rats was significantly increased, which was down-regulated by resveratrol treatment.
*P<0.05 vs. control group; $^{\#}$P<0.05 vs. BLM group (n=8) in each group. BLM: Bleomycin; Res: Resveratrol; CON: control.

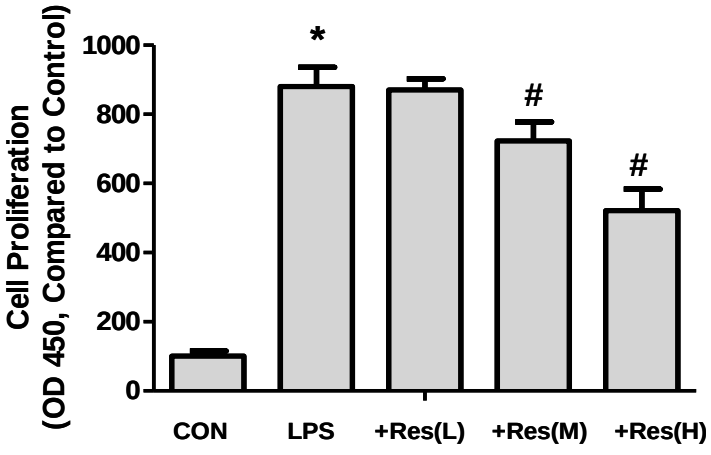

Figure 2. Effect of resveratrol on pulmonary fibroblast proliferation. Cells were cultured for 48 h and cell proliferation was determined by BrdU Marking. LPS (10 ng/ml) incubation stimulated a significant proliferation of pulmonary fibroblast. Resveratrol (10, 50, and 100 μM) pre-incubation inhibited the proliferation does-dependently.
*P<0.05 vs. control group; $^{\#}$P<0.05 vs. LPS group (n=4). Res: Resveratrol; Res: Resveratrol; CON: control; LPS: lipopolysaccharide.

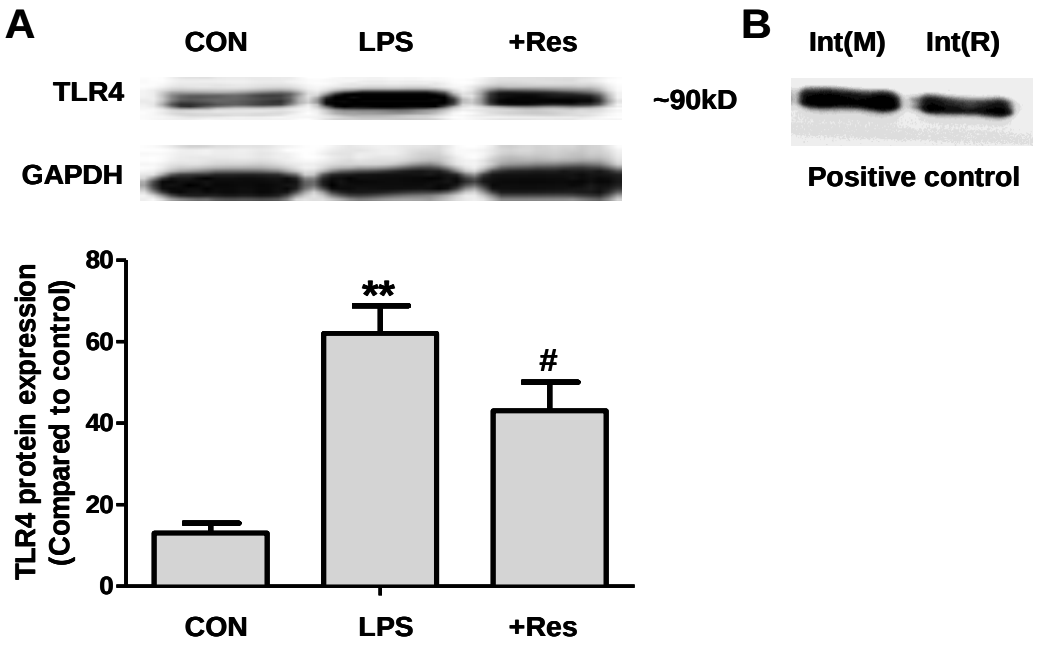

Figure 3. TLR4 expression in lung tissues. To observe the activation of TLR4 signaling, lung tissues were collected from control rats, BLM injected rats and resveratrol treatment received rats. Then TLR4 protein expression was determined using western blot. (A) The protein expression of TLR4 in lung tissue from BLM injected rats was significantly increased while resveratrol treatment decreased TLR4 level. (B) TLR4 protein expression in intestine tissue from mouse and rat, which is a positive control for TLR4 antibody.
*P<0.05 vs. control group; #P<0.05 vs. LPS group (n=8) in each group. Int: Intestine; BLM: Bleomycin; Res: Resveratrol; CON: control; LPS: lipopolysaccharide.

was cultured with LPS with or without resveratrol for 24 h. Proteins were collected and detected by western blot. Nuclear proteins were collected for EMSA experiment. The result showed that LPS (10 ng/ml) incubation significantly up-regulated the expression and activity of NF-κB, which was inhibited by resveratrol (Figure 6).

DISCUSSION

In the present study, we examined the effect of resveratrol on LPS-induced pulmonary fibroblast proliferation, and demonstrated the critical role of TLR4 signaling pathway in the process. *In vivo* date showed that plasma level of inflammatory factors such as IL-1, IL-6 and tumor necrosis factor (TNF)-α were all elevated in BLM-induced PF rats. In cultured pulmonary fibroblast, LPS incubation caused cellular proliferation and activation of TLR4-NF-κB pathway which were reversed by resveratrol. Pharmacological inhibition of NF-κB abolished the effect of LPS and resveratrol on pulmonary fibroblast proliferation.

Pulmonary fibrosis is characterized by interstitial change, it also usually be the final stage of interstitial lung disease. The pathophysiological mechanism of PF has not been fully revealed. But inflammation was thought as an important characteristic of pulmonary fibrosis and also a major reason of pulmonary fibroblast proliferation (Bringardner et al., 2008). Inflammation and the initial immune response induce secretion disorder of cytokines and chemokines. It was demonstrated that inflammatory factors such as IL-1, IL-6, monocyte chemoattractant protein-1 (MCP-1) and TNF-α were significantly increased in PF patients and several PF animal models (Gasse et al., 2011; Oikonomou et al., 2006). Similar situation was observed in our research as the plasma level of IL-1 and IL-6 were all elevated in BLM-induced PF rats. The up-regulation of these inflammatory factors both in blood and lung tissue suggested that inflammation is not only a local response but also a systemic change in PF. Secretion disorder of cytokines and chemokines always caused a series of structural changes in lung. Uncontrolled proliferation of pulmonary fibroblast was one of the changes. Pulmonary fibroblast replaced the position of alveolar epithelial cells, decreased the lung compliance and hence respiratory function (Davies and Richeldi, 2002). It was also able to cause the imbalance of extracellular matrix (ECM), stimulating deposition of connective tissue, collagen, etc. Ultimately, the structural changes induce a persistent change of respiratory function (Taniguchi et al., 2010). Here, in the present study, we observed the dramatically proliferation of pulmonary fibroblast induced by LPS *in vitro*, while resveratrol inhibited the proliferation strongly which suggested a therapeutically

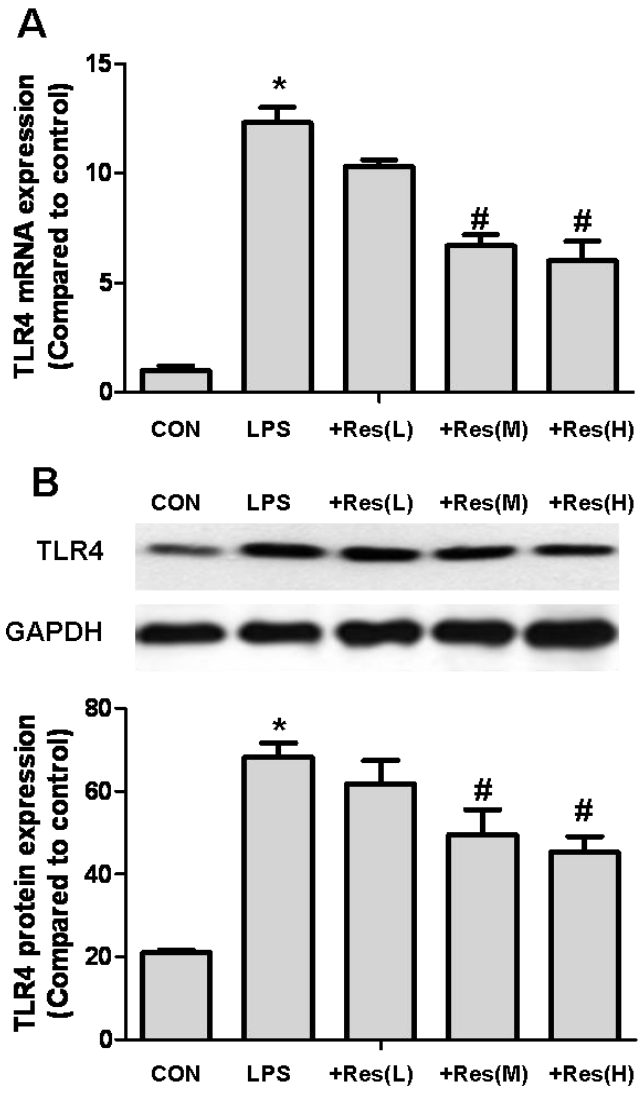

Figure 4. Effect of LPS and resveratrol on TLR4 expression in pulmonary fibroblast. We investigate the direct effect of LPS and resveratrol on TLR4 expression in cells. Pulmonary fibroblast were cultured with LPS with or without resveratrol for 24 h. RNA and protein were collected and detected by real-time PCR and western blot, respectively. (A) LPS (10 ng/ml) incubation significantly increased TLR4 mRNA expression which was inhibited by resveratrol in dose-dependent way. (B) Western blot showed the similar changes of TLR4 protein expression. TLR4 expression was induced by LPS but significantly inhibited by resveratrol in dose-dependent way. *P<0.05 vs. control group; #P<0.05 vs. Lipopolysaccharide (LPS) group (n=4). Res: Resveratrol.

role of resveratrol in PF treatment.

Inflammation response is a complex process and lots of signaling pathways are involved in it. Previous study demonstrated that TLR plays a critical role during inflammation and innate immune response (Zhou et al., 2009). Damage associated molecular pattern molecules (DAMPs) and LPS are both capable to bind to TLR4 and LPS is thought a natural ligand of TLR4. The response of TLR4 activation induced by LPS is usually transduced via the IL-1 receptor signaling complex, which includes two essential adaptor proteins, myeloid differentiation 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) as well as the IL-1 receptor-associated kinase (IRAK). There is also MyD88-independent pathway such as TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) mediating the action, but NF-κB is recognized as the downstream common mediator of TLR4 signaling pathway (Apetoh et al., 2007; Lu et al., 2008). NF-κB plays as a master switch transactivating LPS's pro-inflammation action. It stimulates the expression of the pro-inflammatory cytokines IL-1, -6, and -8 (Takeuchi et al., 2001). It is demonstrated that TLR4 is generally expressed in various lung epithelial cells and stromal cells such as alveolar epithelial cells, bronchial epithelial cells and vascular endothelial cells. TLR4/NF-κB pathway is involved in airway inflammation and other pulmonary inflammatory disease (Perros et al., 2011). In the present study, we observed elevated expression of TLR4 in lung tissue from PF rats, and *in vitro* experiment showed that LPS incubation activated TLR4/NF-κB pathway in pulmonary fibroblast. Furthermore, LPS induced proliferation of pulmonary fibroblast was abolished after NF-κB inhibition by NF-κB inhibitor BAY 11-7085. But the proliferation of cells was not back to normal level, implying that we cannot exclude other pathway mediating the function of TLR4 except for NF-κB. In fact, IRF3 and MAPK pathway could also be activated by TLR4, which is involved in inflammation regulation (Patel et al., 2011). All these results indicated an important role of TLR4 signaling pathway during inflammation in PF and suggested the critical role of NF-κB in mediating pulmonary fibroblast proliferation in PF. Utilization of NF-κB inhibitor *in vivo* and gene knockout animal would be helpful to reveal the exact role of TLR4/NF-κB pathway in PF.

Resveratrol is primarily extracted from grapes and other plants. It is abundant in red wine. Lots' of studies have revealed that resveratrol play a protective role in cardiovascular diseases (Bradamante et al., 2004). Recent study also indicated a role of resveratrol in PF, but the underlying mechanism remains unknown. The effect of resveratrol involves several aspects, including antioxidant effect, cyclooxygenase (COX) inhibition, peroxisome proliferator-activated receptor (PPAR) activation, endothelial nitric oxide synthase (eNOS) induction, silent mating type information regulation 2 homolog 1 (SIRT1) activation, etc (Csiszar, 2011; Lagouge et al., 2006; Li et al., 2010). The expansive effect of resveratrol makes it not to only play a protective role in cardiovascular disease, research during past decades have revealed that resveratrol plays an important role in cancer, diabetes, connective tissue disease, etc (Aggarwal et al., 2004; Szkudelska and Szkudelski, 2010; Elmali et al., 2007). Recently, animal experiments have indicated that resveratrol is able to attenuate and even reverse the established PF, but the underlying mechanism is still

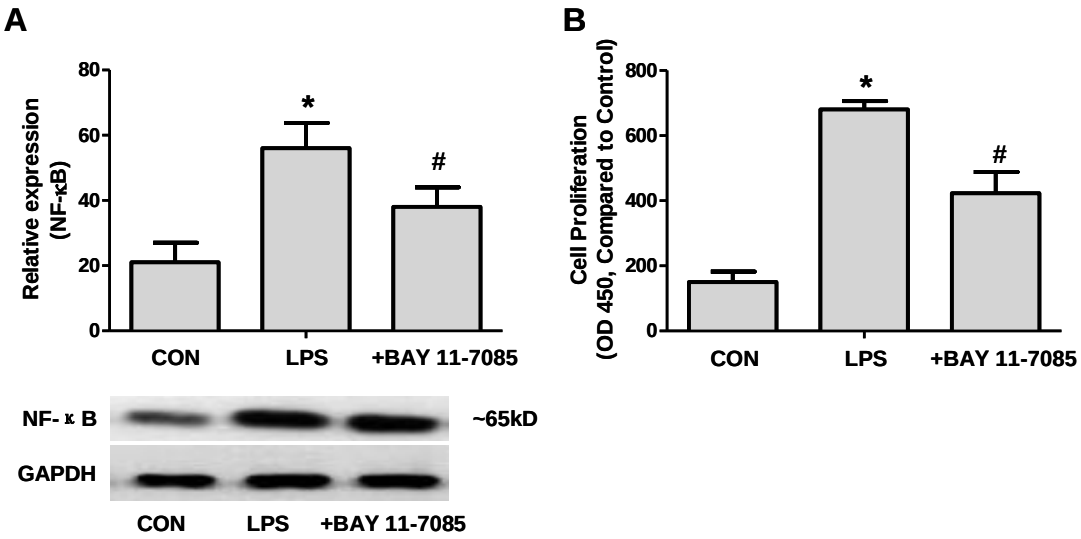

Figure 5. NF-κB inhibiion cancel the effect of LPS and resveratrol on pulmonary fibroblast proliferation. Furthermore, to investigate the role of NF-κB in proliferation regulation by LPS, BAY 11-7085, a NF-κB inhibitor was used. We found that pre-treatment of BAY 11-7085 cancelled the pulmonary fibroblast proliferation induced by LPS, which suggest a critical role of NF-κB in pulmonary fibroblast proliferation regulation. *P<0.05 *vs* control group; #P<0.05 vs. Lipopolysaccharide (LPS) group (n=4).

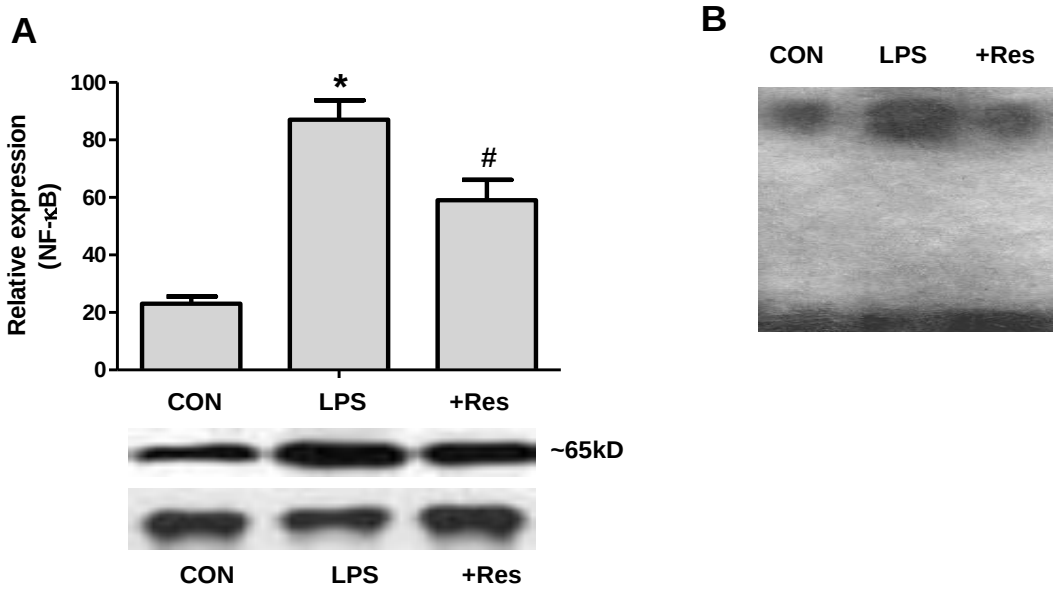

Figure 6. Effect of LPS and resveratrol on NF-κB expression and activity in pulmonary fibroblast. We investigate the direct effect of LPS and resveratrol on NF-κB expression and activity in cells. Pulmonary fibroblasts were cultured with LPS with or without resveratrol for 24 h. Proteins were collected and detected by western blot. Activity of NF-κB was determined by EMSA. (A) LPS (10 ng/ml) incubation significantly increased NF-κB expression which was inhibited by resveratrol. (B) NF-κB was activated by LPS (10 ng/ml) incubation and resveratrol pre-treatment significantly inhibited the activation of NF-κB. *P<0.05 vs. control group; #P<0.05 vs. Lipopolysaccharide (LPS) group (n=4). Res: Resveratrol.

seldom known (Akgedik et al., 2012).

In the present study, resveratrol treatment down-regulated plasma level of inflammatory factors *in vivo*. Cell culture experiment showed that resveratrol inhibited the activation of TLR4/NF-κB pathway induced by LPS in pulmonary fibroblast. All these results prompted that anti-inflammatory effect of resveratrol may contribute its therapeutic role in PF treatment. Sener et al. (2007) found that

found that in BLM-induced lung injury rats, resveratrol treatment decreased TGF-β, Il-1, Il-6 and TNF-α level. There were also several experiments that suggested the effect of resveratrol on TLR4 signaling pathway. Research from Capiralla et al. (2012) demonstrated that resveratrol mitigated microglial inflammation by inhibiting the TLR4/NF-κB/STAT signaling cascade. But how resveratrol affected TLR4 signaling pathway remains unclear and opposite opinions exist. Someone thought the function was dependent on MyD88 pathway while a few research said it did not. Different mechanism may attribute to different tissue and cell types. Deeper investigation is needed to finally answer the question of how can resveratrol interact with TLR4 signaling pathway in pulmonary fibroblast.

The present study investigated the role of resveratrol in PF treatment. We demonstrated the anti-inflammatory effect of resveratrol and its effect on TLR4 signaling pathway. There is still a long way to go for therapeutical utilization of resveratrol. Anyway, the precious constituent of red wine, has been more and more attractive for new drug development. Several structural modifications have been made for avoiding the weakness of resveratrol including low water-solubility, short half-time *in vivo*, etc. Further research will push the potential candidates to a light future in cardiovascular disease and other diseases treatment.

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Full Length Research Paper

Elderly drug utilization in the community assessed through pharmacy dispensing data

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People of 65 years and above now comprise a greater share of the world's population than ever before, and this proportion will increase during the 21st century. In Spain, between 55 and 90% of the elderly consume a drug. This study characterizes the use of drugs by elderly through dispensing data at the community pharmacy. This study was conducted at a community pharmacy in Madrid, Spain in 2011. A retrospective and descriptive consumption study was conducted using computerized pharmacy dispensing records for all pensioner patients. Anatomical Therapeutic Chemical (ATC) Classification code of all drugs dispensed was recorded in this database accordingly and this classification was used. The 10 most widely used ATC subgroups (2nd level) were determined. These most widely used ATC subgroups were examined using ATC-codes of the 5th level, thus mostly consumed drugs were estimated. A total of 40, 177 drugs were dispensed to patients with prescriptions for pensioners. Anti-inflammatory and analgesic were by far the most widely used drugs: 37.2% of all elderly used drugs from this subgroup. The use of drugs from the remaining nine subgroups was considerably lower, ranging from 9.0% (drugs for obstructive airway diseases) to 4.5% (antineoplastic and beta blocking agents). Cardiac therapy and psycholeptic were used by 7.8%. Diuretic were used by 7.5% of elderly people, while antibacterial for systemic use and psycoanaleptic were used by 5.6%. Psychoanaleptics was consumed in 5.6%, mostly represented by venlafaxine and citalopram. According to the dispensing data, drug use in this sample is similar to that reported by other studies conducted in Spain and abroad. Majority of the elderly were exposed to anti-inflammatory, analgesic and drugs for obstructive airway diseases. Other ATC-subgroups for treatment of cardiovascular conditions were used. This study demonstrates the need for involvement of pharmacists to ensure efficacy and safety in the use of drugs by sensitive populations such as elderly at the community setting.

Key words: Community pharmacy, elderly, drug use, Spain.

INTRODUCTION

People of age 65 and above now comprise a greater share of the world's population than ever before, and this proportion will increase during the 21st century (Morchadze et al., 2009). It is projected that the elderly population of the world will cross the one billion mark by the year 2020. By that time, over 700 million old people will be living in developing countries (Hutton, 2008).

According to Abellán and Ayala (Abellán and Ayala, 2012), 17.4% of the Spanish population are 65 years or above with 98% of these persons living in their main family home, that is, ageing at home. The most prominent sociodemographic characteristics are the number of very elderly (43% are 75 or above), number of women (almost 6 out of every 10 are women), number of widows or widowers

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(31%), low educational level (44% have no education), and the predominance of pensioners (76%). The high number of widows/widowers and single people explains the significant proportion of elderly people living alone (20%), an aspect which mainly affects women and the very oldest.

A study on drug consumption in Spain shows that between 55 and 90% of the elderly consume at least one drug (Ruíz, 2006). The average number of drugs in the non-institutionalized elders is between 2 and 4 drugs per person per day, with an average of 2.6, the proportion being larger in the pool of women. This consumption takes place over prolonged periods of time, with a high degree of dependence on others for taking medication and with high rates of self-medication. A study about drug prescribing and use among elderly people in Spain showed that drug use is highly prevalent among the elderly, that many medicines without any demonstrated benefit are being taken, and that potentially harmful drugs were being used by a high proportion of patients without medical follow-up (Mas and Laporte, 1983). Another study showed a high prevalence of multiple medications and a high percentage of elderly patients using inappropriate medications (35%). The same study showed that 77.7% of the inappropriate prescriptions originate from the family doctor, which is, therefore, a challenge for the primary care clinic (Moral et al., 2006). The results of the study could help to improve the medicine use among elderly, specifically at the community setting, from the knowledge of medicines more used by them.

Furthermore, Spain has many community pharmacies, but there is little pharmacy practice research (Gastelurrutia et al., 2005). “The existence of authors who publish very few studies and the high insularity index observed in the articles may be considered as negative indicators for community pharmacy-based research in Spain” (Iglesias et al., 2007). Therefore, this study was conducted to characterize the use of drugs by the elderly through examining dispensing data in one community pharmacy.

METHODOLOGY

Setting

A prospective and descriptive research study was conducted over an 11-month period (October 2010 August, 2011) in a community pharmacy in Madrid, Spain. The community pharmacy is a shift of 12 h, attached to Ambulatory Health Center, which dispenses about 4000 prescriptions each month. Like all community pharmacy in Spain, this is a private community pharmacy.

Data source

The study was performed with pharmacy dispensing data from the Unycop Win database, which is an Office Pharmacy Management program in Spain developed by Unycop Pharmacy Group (Unycop, 2012). More than 4,200 pharmacies in Spain are computerized with this software. Unycop Win has visual and intuitive applications integrated with the latest tools from Microsoft; a Menu Bar and "Toolbar Icons" that provide access to the entire application; and is adapted to the legal and administrative requirements of each region and fully integrated with *Bot Plus*, the most comprehensive database of pharmaceutical knowledge in Spain. This database comprises all prescriptions of all patients served at the community pharmacy, regardless of reimbursement status.

Inclusion/exclusion criteria

All drugs registered in the database and dispensed during the study period under code number 3 (corresponding to pensioners) were included in the study. Non-drug agents and accessories which require a medical prescription were excluded.

Data analysis procedures

The following elements from Unycop Win database were considered: drug name, therapeutic class, and units dispensed. The Anatomical Therapeutic Chemical Classification (ATC) code of all drugs dispensed was recorded in Unycop Win database, and accordingly, this classification was used in this study. In the ATC classification system given by World health Organization (WHO), the drugs are divided into different groups according to the organ or system on which they act and their chemical, pharmacological and therapeutic properties (Rønning, 2001). Drugs are classified in groups at five different levels. The drugs are divided into fourteen main groups (1st level), with two therapeutic/pharmacological subgroups (2nd and 3rd levels). The 4th level is a therapeutic/ pharmacological/chemical subgroup and the 5th level is the chemical substance.

For example, the code N02BE01: the first character (N) represents the main anatomical group. In this example, N = Nervous System. Characters two and three (02) represent the therapeutic subgroup. In this example, N02 = Analgesics. Character four (B) represents the pharmacological subgroup. In this example, N02B = Other analgesics and antipyretics. Character five (E) represents the chemical subgroup. In this example, N02BE = Anilides. Characters six and seven (01) represent the chemical substance. In this example, N02BE01 = Paracetamol (acetaminophen).

Collected data were recorded in a database that was processed in Microsoft Access. The 10 most widely used active substances were determined using ranking the percentage. These most widely used ATC subgroups were examined using ATC-codes of the 5th level, allowing estimation of the most commonly used drugs.

Approval from community pharmacy owner was obtained. Only data related to drugs sold was used. Patient data is not registered in the Unycop Win database, because this pharmacy does not have the licenses granted by the Spanish Data Protection Law for this registers. Therefore, the ethical approval was not considered.

RESULTS

Table 1 shows the ATC subgroups that were most widely used by elderly according to dispensing data and the distribution of active substances used by elderly. During the study period, a total of 40 177 drugs were dispensed. Anti-inflammatory and analgesic drugs were by far the

Table 1. Distribution of active substances used by elderly, according to ATC Classification, (n = 40 177)

ATC classification[a]	n	Percentage within the subgroups	Percentage within the total
M01AE01 Ibuprofen	7 203	48.1	17.9
M01AC01 Piroxicam	3 525	23.5	8.7
R03CC02 Salbutamol	2033	49.8	5.0
R03AK06 Salmeterol	1856	45.5	4.6
C08CA01 Amlodipine	1348	37.1	3.3
C03AB03 Hydrochlorothiazide	1289	42.6	3.2
N05CF02 Zolpidem	1233	38.8	3.0
C01DA02 Glyceryl trinitrate	1145	36.0	2.8
N02BA01 Acetylsalicylic acid[b]	1022	6.8	2.5
J01CR02 Amoxicillin	995	43.9	2.4
N02BE01 Paracetamol	906	6.0	2.2
C01AA05 Digoxin	856	26.9	2.1
N05BA01 Diazepam	846	26.6	2.1
C08DA51 Verapamil	820	22.6	2.0
J01FA10 Azithromycin	791	34.9	1.9
C03DA01 Spironolactone	698	23.0	1.7
N06AX16 Venlafaxine	714	31.5	1.7
C07BB03 Atenolol	587	32.3	1.4
L01BA01 Methotrexate	533	29.4	1.3
N06AB04 Citalopram	460	20.3	1.1
C07AG02 Carvedilol	361	19.9	0.8
L01AA01 Cyclophosphamide	358	19.7	0.8

[a]Active substances showing an example of the two most used drugs within each of the subgroups consumed. [b]Concentration of tablet was not considered in the classification.

most widely used: 37.2% of all elderly used drugs from this subgroup. Within this subgroup, paracetamol was by far the most widely used analgesic followed by acetylsalicylic acid, ibuprofen, and piroxicam.

The use of drugs from the remaining nine subgroups was considerably lower, ranging from 9.0% (drugs for obstructive airway diseases) to 4.5% (antineoplastic and beta blocking agents). Within the subgroup “drugs for obstructive airway diseases,” salbutamol and salmeterol were the most commonly used while methotrexate and cyclophosphamide were the most frequently prescribed antineoplastic drugs. Atenolol and carvedilol were the most commonly used beta blocking agents.

Cardiac therapy (C01) and psycholeptics (N05) were used by 7.8%. Diuretics (C03) were used by 7.5% of the elderly, while antibacterials for systemic use and psychoanaleptics were used by 5.6%. The most common drugs dispensed within the subgroup of diuretics were hydrochlorothiazide and spironolactone. Digoxin and glyceryl trinitrate were widely used within the cardiac therapy subgroup.

The ATC subgroup of calcium channel blockers was largely represented by the use of amlodipine and verapamil. The ATC subgroup of psycholeptics appeared to be a heterogeneous group represented by drugs like zolpidem and diazepam. The subgroup of psychoanaleptics was taken by 5.6% of the elderly and the most common members were venlafaxine and citalopram. Finally, the antibacterials for systemic use subgroup was used by 5.6% with the most common representatives being azithromycin and amoxicillin.

DISCUSSION

This study examined the pattern of medication use among the elderly in a community pharmacy in Madrid. The 10 most widely prescribed drugs were anti-inflammatory drugs, analgesic drugs, and drugs for obstructive airway diseases. Non-steroidal anti-inflammatory drugs (NSAIDs) were widely used within the analgesics+anti-inflammatory subgroup. Similar results were presented by Pilotto et al. (2003), who identified the prevalence of specific drug use in elderly outpatients and the relationship between NSAID use and gastrointestinal disturbances and therapies in elderly subjects treated by their general practitioner (GP). Other studies have also shown that NSAIDs are prescribed to a great extent in elderly patients (Visser et al., 2002; Hogan et al., 1994; Rahme, 2001).

These results could be associated with the high prevalence of chronic pain in the Spanish general population (Català et al., 2002). General chronic pain prevalence in Spain was estimated at 31.4% in women and 14.8% in men, the most frequent causes being osteoarthritis/arthritis and rheumatoid arthritis, results epidemiologically similar to those reported for other European countries such as the United Kingdom, Ireland, Italy, Norway and Belgium (Breivik et al., 2006).

The burdens of chronic obstructive airway diseases among the elderly in Europe and worldwide are increasing (Lundba and Gulsvik, 2003). Of the general Spanish population between 40 and 80 years of age, 10.2% suffer from obstructive airway diseases (Soriano et al., 2010). Obstructive airway diseases in Spain generate approximately 10 to 12% of family doctor visits. Similarly, these diseases cause 35 to 40% of visits to pulmonologists and cause 35% of permanent work disability (Echave-Sustaeta and Villena, 2002). This justifies the use of medications such as salbutamol and salmeterol reported in this study.

According to studies developed by Wolf-Maier and Cooper, (2003), Europe should be considered a high prevalence hypertension region. In Germany and Finland, roughly 44% of the adult population can be diagnosed with hypertension (HT) (Wolf-Maier and Cooper, 2003). Cardiovascular diseases constitute the first cause of death in the whole Spanish population. Both cerebrovascular disease and ischemic heart disease together account for 60% of global cardiovascular mortality (Redón et al., 2007). The prevalence of essential hypertension in the Spanish population more than 60 years of age is greater than 65% (Sierra et al., 2008). Beta blocking agents, for example, showed a low consumption when compared with other subgroups in this study. These data are consistent with those reported in other studies of beta blockers consumption which also reported decreasing consumption with age and other pathological factors in the elderly (Garcia-Molla et al., 2011). At the same time, this result could support the hypothesis that beta blockers do not offer much additional benefit to the control of other risk factors and prevent the progression of target organ damage, so that they are relegated to specific indications (Protocolo de HTA, 2011; Che et al., 2009).

Likewise, cardiac therapy (ATC- group C01) showed considerable consumption; this result is similar to that of Straand and Rokstad (1999), where nitrates and digitalis preparations were more representative drugs. Other studies showed the same tendencies (Loyola et al., 2011; Rozenfeld, 2008).

Our calcium channel blocker (ATC-group C08) consumption results are consistent with other studies developed at the primary care level (Mendes-Netto et al., 2011). This result, however, conflicts with the findings of the PROTECT project, where calcium channel blockers were used in 97.8% of all the cases in the outpatient sector (Ballarín et al., 2012). Taken together, the results related to drugs active on the cardiovascular system are consistent with the European Society of Cardiology/ European Society of Hypertension (ESC/ESH) guidelines, which recommend that thiazide diuretics should be considered just as suitable as β-blockers, calcium antagonists, angiotensin-converting-enzyme (ACE) inhibitors, and angiotensin receptor blockers for the initiation and maintenance of antihypertensive treatment (Grossman and Verdecchia and Micheli, 2011). Similarly, psycholeptics were the drugs most used by the elderly according to some current studies (Tomàs, 1999; Ramage-Morin, 2009).

According to the Spanish Agency for Medicines and Health Products, Spain is the second leading European Union country in antibiotic consumption after France, even though the use of these drugs has been reduced from 22.8 doses per 1,000 inhabitants/day in 1995 to 19.8 in 2007 (Lázaro, 2008). The antibiotic consumption reported in this study is consistent with these findings.

The findings related to antineoplastic agents (ATC-subgroup L01) is consistent with cancer prevalence, because of the overall aging of the population and the fact that cancer incidence and mortality rises exponentially in the 50 to 85 year old age groups (Nathan et al., 2006). This pathology in the elderly represents an increasing load for the community, particularly in France, Italy and Spain due to the ageing population in these countries (Verdecchia, 2002). The epidemiological data could support the consumption reported in this study for antineoplastic agents. Similarly, cyclophosphamide and methotrexate are drugs sold in oral dosage form at the community pharmacy. These drugs have been widely used as therapy to treat breast and prostate cancer (Colleoni et al., 2006).

Interestingly, these results showed the use of drugs that, as recommended by Elliott (2006), should be avoided in elderly, namely, benzodiazepines and NSAID. According to these results, future researches will be necessary to address these issues, and a geriatric pharmaceutical care program could be viable to improve the coordination of pharmaceutical treatments, ensuring the safety of pharmacotherapy in elderly patients.

STUDY LIMITATIONS

The limitation of this study is that the consumption of drugs was estimated using a dispensing register. When using a dispensing register for investigating the consumption of a drug, it must be taken into consideration that the register contains information on redeemed prescriptions and not on the actual consumption of drugs by the persons who redeem the prescriptions. The fact that there is no assurance that persons ingested the drugs that they collected at the pharmacy is a limitation.

This limitation has also been recognized in other studies using dispensing data for investigating use of a drug (Sundell et al., 2011; González et al., 2004).

Another limitation is that the database is used to record dispensing and manage cash control, but says nothing about the quality of prescribing. Patient records are not considered in this database; therefore, it was not possible to estimate consumer population. Diagnostic data are not considered in this database; therefore, statements about the appropriateness of drug use cannot be made. These limitations are consistent with the statements of González et al. (2004): the pharmacy databases are designed for administrative control ("PharmacyManagement") rather than for clinical use.

Conclusion

According to the dispensing data, drug use in this sample is similar to that reported by other studies conducted in Spain and abroad. Majority of the elderly were exposed to anti-inflammatory and analgesic drugs and drugs for obstructive airway diseases. Other ATC subgroups for the treatment of cardiovascular conditions were used. This study shows that the Unycop Win database is a valuable source for information on drug utilization, permitting the registration and classification of drugs dispensed in accordance with the ATC Classification System. This study shows the need for involvement of pharmacists to ensure effectiveness and safety for the use of drugs in sensitive populations such as the elderly in community settings.

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Full Length Research Paper

Repaglinide as a safe alternative against hypoglycemia in fasting elderly diabetic patients: A single blinded, placebo-controlled, six period, cross-over study

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Elderly are more prone to develop hypoglycemia or miss meals. Repaglinide and nateglinide are suggested to have glucose-dependent insulinotropic effect. The aim of the present work was to test the effect of both drugs on serum glucose and insulin levels in fasting elderly diabetic patients. Eight elderly diabetics underwent a fixed dose single blinded, placebo controlled, six period, cross-over study at the Department of Geriatrics and Gerontology - University hospital. Patients received either repaglinide 2 mg, nateglinide 120 mg or a placebo, both under fasting and non-fasting states. Serum glucose and insulin were measured at 30 min intervals for four hours, following drug or placebo administration. None of the eight patients developed hypoglycemia under the fasting state in response to repaglinide and only one patient developed mild hypoglycemia (3.7 mmol/L) under the fasting state in response to nateglinide. Area under the serum insulin concentration-time curve was significantly lower (p = 0.039) in the fasting state, compared to the non-fasting state, in response to repaglinide, but not to nateglinide. The present study suggests that in case of elderly diabetic patients, who may miss meals, repaglinide is a safe alternative to other antidiabetics. As for nateglinide, further studies are required.

Key words: Repaglinide, nateglinide, hypoglycemia, fasting, elderly.

INTRODUCTION

Type 2 diabetes is a major public health problem especially in elderly. Its prevalence in elderly, in the USA, is more than double its prevalence in the adult age group (National Diabetes Information Clearinghouse). Treatment decisions for this age group are particularly influenced by age and life expectancy, comorbid conditions and severity of the vascular complications. Furthermore, adherence to life style modification may be compromised in the elderly due to comorbid conditions and psychosocial limitations (Rosenstock, 2001). Elderly subjects may skip meals (Posner et al., 1993), and those with poor memory are more than twice as likely to do so (Perkins et al., 1999). This increases the risk of hypoglycemic coma that is proved to be increased in elderly frail patients (Ben-Ami et al., 1999). Changes in pharmacokinetics that occur in the elderly also lead to potential adverse effects and drug interactions and therefore, should be considered when selecting pharmacological therapy (Rosenstock, 2001).

Repaglinide and nateglinide are short acting non-sulfonylurea insulin secretagogues of the meglitinides group, that are considered of lower hypoglycemic risk (Miwa et al., 2004; Meneilly, 2011). Hu et al. (2001) reported that nateglinide showed a glucose-dependent insulinotropic effect *in-vitro*, while repaglinide failed to demonstrate such effect. On the other hand, repaglinide resulted in a blunted β cell insulin secretory response in healthy subjects during euglycemic and modest clamp

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Table 1. Demographic characteristics of the patients.

Characteristic	Value
Gender (female/male)	6/2
*Age (years)	63.1 (60 - 75)
* Body mass index (Kg/m^2)	35 (29 - 40)
*Known duration of diabetes (years)	1.9 (0 - 5)
*HbA1c(%)	6.1 (5.0 - 7.48)
*ALT (IU/L)	27.1 (9 - 60)
*AST (IU/L)	31.2 (17 - 77)
*Serum creatinine (micromol/l)	76.25 (35.4 -256.4)
*#Estimated creatinine clearance (ml/min)	135.8 (36.4 - 247.9)
Co-morbidities (N)	
Hypertension	2 (controlled on enalapril)
Ischemic heart disease	1 (on nitroglycerin and aspirin)
Renal impairment	1 (S.Cr. 256.4 µmol/L; Estimated creatinine clearance 36.4 ml/min)
Chronic liver disease (AST, ALT)	1 Hepatitis C virus (AST 60 IU/L, ALT 77 IU/L); 1 Portal hypertension (AST 21 U/L, ALT 29 U/L)

* Data are presented as means (range). #Estimated Creatinine clearance was calculated using Cockroft Gault formula.

studies (Aldhahi et al., 2004). These results suggest a low risk of hypoglycemia for both drugs, even if they were administered without meals.

However, differences might exist between the controlled hypoglycemia conditions produced in clamp studies and real world hypoglycemia in patients using insulin secretagogues (Aldhahi et al., 2004). This is a special concern in elderly, where a progressive loss in reserve capacity affects the endocrine system, with loss of homeostatic regulation (Gruenewald and Matsumoto, 2009). Besides, in another study, C-peptide levels were estimated during a hyperglycaemic and an euglycaemic clamp in Type 2 diabetic patients after repaglinide or placebo. Though C-peptide concentrations were lower after repaglinide administration in the normoglycaemic state than in the hyperglycaemic one they remained higher than after placebo (Rudovich et al., 2004). In addition, plasma insulin levels rose following nateglinide administration before glucose injection in recently diagnosed type 2 diabetes patients (Whitelaw et al., 2000).

The effects of repaglinide and nateglinide on blood glucose and serum insulin levels have never been measured in the fasting state. Although the effect of omitting lunch on blood glucose level was studied in patients on repaglinide treatment, no repaglinide dose was administered (Damsbo et al., 1999). The aim of the present work was to study the safety of repaglinide and nateglinide, if the patient missed or delayed his meal by estimating their effect on blood glucose and serum insulin levels in the fasting state.

METHODOLOGY

Study protocol

The study was approved by the Research Ethics Committee of the faculty of medicine, Ain Shams University, Cairo, Egypt. The investigation was carried out in accordance with the Declaration of Helsinki. Written informed consents were obtained from all participants.

Subjects

Nine Egyptian Type 2 diabetic elderly subjects (≥ 60 years) were recruited for the study. Eight completed the study and one subject dropped out after one day for personal reasons (did not tolerate the multiple needle pricks). Demographic characteristics of the patients are shown in Table (1). Three of the eight subjects that completed the study were previously treated with metformin and five were newly diagnosed. Exclusion criteria were: current use of insulin secretagogues, fasting blood glucose above 15 mmol/L, late complications of diabetes or severe concurrent disease. Since comorbidities are common in elderly patients, patients with stable comorbidities that did not represent a contraindication to either drug were not excluded from the study.

Study design

This study was a fixed dose, single blinded, placebo controlled, six period, cross-over study. All patients underwent comprehensive geriatric assessment and laboratory evaluation before the beginning of the study. Metformin morning dose was skipped on the days of the study. Each patient was studied for six non-consecutive days (three days on the fasting protocol and three on the non-fasting protocol). On the mornings of the tests, patients were admitted to the Geriatrics and Gerontology Department at Ain Shams University Hospital. For the non-fasting protocol, two baseline venous samples were collected at 30 min interval, starting at 8:30 am. Immediately afterwards, repaglinide 2 mg (Novonorm, Novo Nordisk, Manufactured by Boehringer Ingelheim Pharma GMbH & Co. KG, Ingelheim am Rhein, Germany), nateglinide 120 mg (Starlidine, International Drug Agency for Pharmaceutical Industries, Port Said, Egypt) or a placebo (Alexandria Company for Pharmaceuticals and Chemical Industries, Egypt) were administered to the patient, followed by an ordinary Egyptian

breakfast composed of bran mixed bread and brown beans (500 kcal, 55% carbohydrate, 30% fat, and 15% protein). Eight venous samples were collected at 30 min intervals afterwards (starting 30 min after meal). Patients were closely observed during the time of study to detect any symptoms suggestive of hypoglycemia. The same procedure was performed for the fasting protocol with the exception of meal serving. During the fasting protocol, capillary blood glucose was checked at 30 min intervals or as needed to early detect any possible hypoglycemia. A glucose level of 3.33 mmol/L was set as a threshold to terminate the sampling and correct hypoglycemia. In case the patient presented with symptoms of hypoglycemia and requested termination of the study, this request was honored, even if blood glucose level was above 3.33 mmol/L. At the end of each day, samples were centrifuged and the serum decanted and stored at -20°C, till they were analyzed. Serum glucose and insulin concentrations were estimated in each sample.

Laboratory determination

Samples were assayed for glucose and insulin at the Clinical Pathology Department at Ain Shams University Hospital, Cairo. Serum glucose was measured using a glucose oxidase method. Serum insulin was measured using enzyme-linked immunosorbent assay (Accu Bind ELISA Microwells, Monobind Inc, Lake Forest, CA). The assay has < 1% cross-reactivity with proinsulin, with no detectable reaction with C-peptide and inter- and intra-assay CV of 5%. The detection range was 35 to 2080 pmol/L. Hemolyzed samples were not included in analysis.

Statistical analysis

The sample size of eight patients was estimated based on the data provided by Rudovich et al. (2004) to detect a 50% difference between insulin levels in the fasting and non fasting state with a power of 80% at the 5% level of significance. Data are expressed as mean ± S.E.M. Significant differences between groups of data were assessed using the paired Student's t-test, and statistical significance was assumed if $P < 0.05$. Area under the curve (AUC) was calculated using graphpad prism (version 3.02). The mean of the two baseline samples was used for comparison with the other time points. There were 12 missing values (2.5%), 10 samples due to hemolysis and two samples because study was terminated for one patient in one day after 3 h. Missing values were substituted by imputed data (calculated by the expectation-maximization method (EM), SPSS version 17.0, SSPS Inc, Chicago, IL, USA).

RESULTS

Effect on serum glucose level

Mean baseline serum glucose levels were not significantly different among the six testing days. The standard meal resulted in an elevation of serum glucose level after placebo administration. This elevation was significant two hours after breakfast ($p = 0.047$) compared to baseline level and to fasting state at the same time point (paired Student's t test). On the other hand, placebo did not induce any significant effect on blood glucose level, when administered in the fasting state (Figure 1A). Both repaglinide (2 mg) and nateglinide (120 mg) administered before breakfast prevented this elevation in serum glucose level. Mean glucose level at each time point -following the administration of either drug- was not significantly different from baseline serum glucose level (Figure 1B and C).

In the fasting protocol, repaglinide did not cause hypoglycemia in any of the eight patients. Serum glucose level was not significantly different between any of the eight time points following drug administration and the baseline level (paired Student's t test). Comparing each time point to the corresponding time point of the non-fasting state did not result in any significant difference (paired Student's t test). The area under the concentration-time curve (AUC_{BL-4h}) was not significantly different either (paired Student's t test). The lowest serum glucose level detected after repaglinide, in the fasting protocol was 4.3 mmol/L.

On the other hand, nateglinide induced one mild hypoglycemic event in the fasting state. The patient experienced tachycardia, sweating and drowsiness, three hours after nateglinide administration. His serum glucose level was 3.7 mmol/L. The study was terminated for this patient on that day, upon his request. Mean serum glucose level (for the eight patients) was slightly, but significantly lower than baseline level at the same time point (1.35 ± 0.96 mmol/L; $p = 0.005$; paired Student's t test; Figure 1C). However, there was no significant difference in the AUC_{BL-4h} between the fasting and non-fasting states.

Effect on serum insulin level

Baseline serum insulin levels were not significantly different on different days. Repaglinide induced a significant increase in serum insulin level when administered before breakfast. This increase was significantly different from baseline level at 1.5, 2.5, 3, 3.5 and 4 h ($p < 0.05$; paired Student's t test; Figure 2A). In the fasting state, however, there was no significant difference between baseline serum insulin level and any of the eight time points following repaglinide administration. Furthermore, there was significant difference between serum insulin level in the fasting and the non-fasting states at 2.5, 3 and 3.5 h following repaglinide administration ($p < 0.05$; paired Student's t test; Figure 2A). The area under the time concentration curve (AUC_{BL-4h}) was significantly different between the fasting and non-fasting state ($p = 0.039$; paired Student's t test; Figure 2B).

Similarly, nateglinide induced an increase in serum insulin level when administered before breakfast. This increase was significantly different from baseline level at 1.5, 2.5, 3.5 and 4 h ($p < 0.05$; paired Student's t test; Figure 2C). In the fasting state, however, there was no significant difference between baseline serum insulin level and any of the eight time points following nateglinide administration. Comparing each time point to its corresponding one in the non-fasting state showed significant difference only at 4 h following nateglinide administration

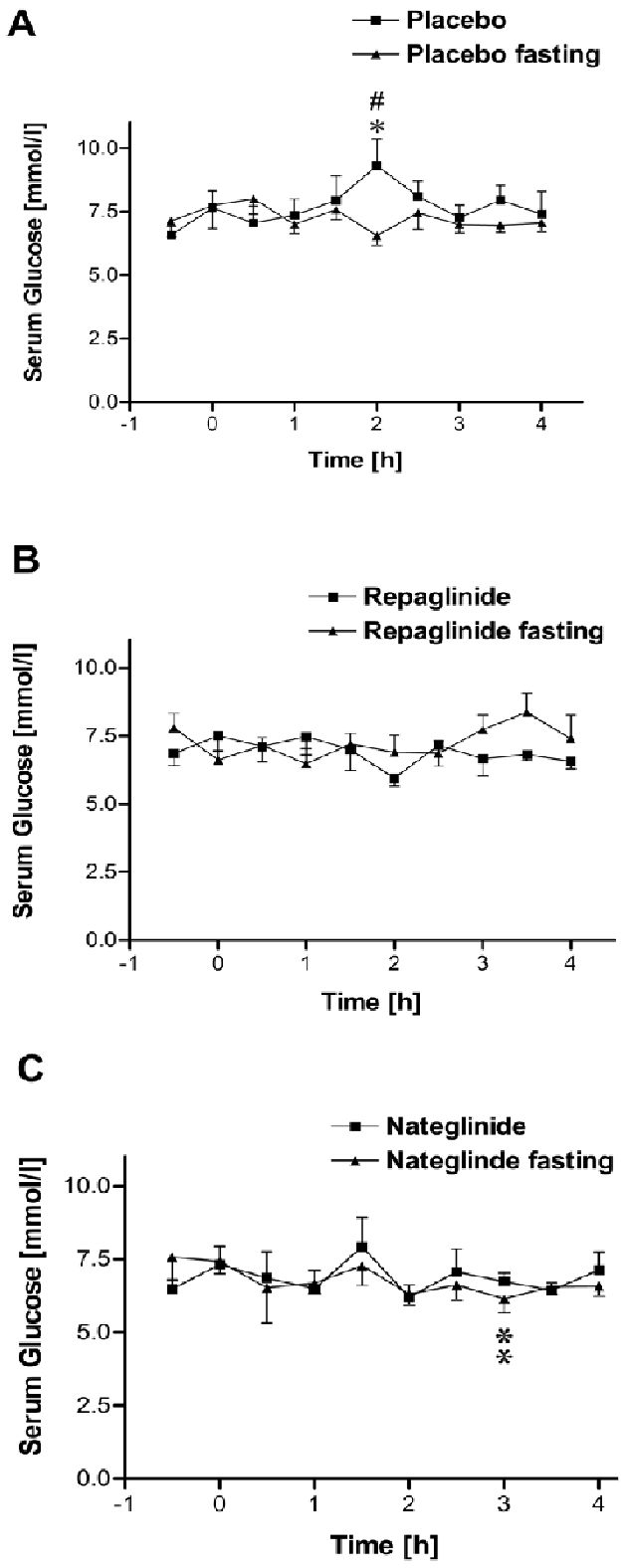

Figure 1. Mean serum glucose concentrations (± SEM) after treatments with a placebo (A), 2 mg repaglinide (B), and 120 mg nateglinide (C) in the fasting and non-fasting state. *indicates $p < 0.05$; **indicates $p < 0.01$ compared to baseline values; #indicates $p < 0.05$ compared to the corresponding time point of the fasting protocol; paired Student's t test.

($p < 0.05$; paired Student's t test; Figure 2C). AUC_{BL-4h} was not significantly different between the fasting and non-fasting states (Figure 2D).

On the other hand, placebo, did not induce a significant increase in serum insulin level compared to baseline levels. However, there was a significant difference between serum insulin levels in the fasting and non-fasting state at 0.5 and 2 h following placebo administration ($p < 0.05$; paired Student's t test; Figure 2E). This resulted in a significant difference in AUC_{BL-4h} ($p = 0.033$; paired Student's t test; Figure 2F).

DISCUSSION

Drug-induced hypoglycaemia is an important consideration when treating diabetes especially in the elderly. This magnifies the importance of nutritional assessment as a part of comprehensive geriatric assessment, as well as considering comorbidities that may influence the pharmacokinetics of the medications used. Medications with lower hypoglycemic risk, particularly when adherence to dietary plans is questionable, may represent a safe option.

Earlier studies suggested a glucose-dependent insulinotropic effect for both repaglinide and nateginide, as mentioned earlier. However, the first adverse effect reported in prescribing information of both drugs is hypoglycemia (Prandin®; Starlix® prescribing information). Further, a systematic review showed that repaglinide and second-generation sulfonylureas conferred similar risks for hypoglycemia (Bolen et al., 2007). Similarly, the overall rate of hypoglycemic events was similar with nateglinide and gliclazide combinations with metformin in a one year-double-blind, double-dummy, multicentre study (Ristic et al., 2007).

The present study was conducted on patients who did not use either drug before, and are therefore at increased risk of hypoglycemia according to data from prescription-event monitoring cohort study that showed a higher incidence of hypoglycemia at the beginning of treatment with nateglinide or repaglinide (Vlckova et al., 2009). However, this adverse effect was not reported during the present study in the non fasting state. In the fasting state though serum glucose level was significantly lower than baseline levels, three hours following nateglinide administration, it did not decrease beyond the hypoglycemic threshold set for this study (3.33 mmol/L). This decrease in serum glucose level was not seen with repaglinide in fasting patients.

The difference in frequency of hypoglycemic events between repaglinide and nateglinide is not consistent in literature. In a multicenter-16 week trial conducted in the US, repaglinide monotherapy induced minor hypoglycemic episodes (blood glucose < 2.78 mmol/L) in 7% of subjects enrolled compared to none of nateglinide treated patients (Rosenstock et al., 2004). In this study, both drugs were initiated at the starting dose and stepwise

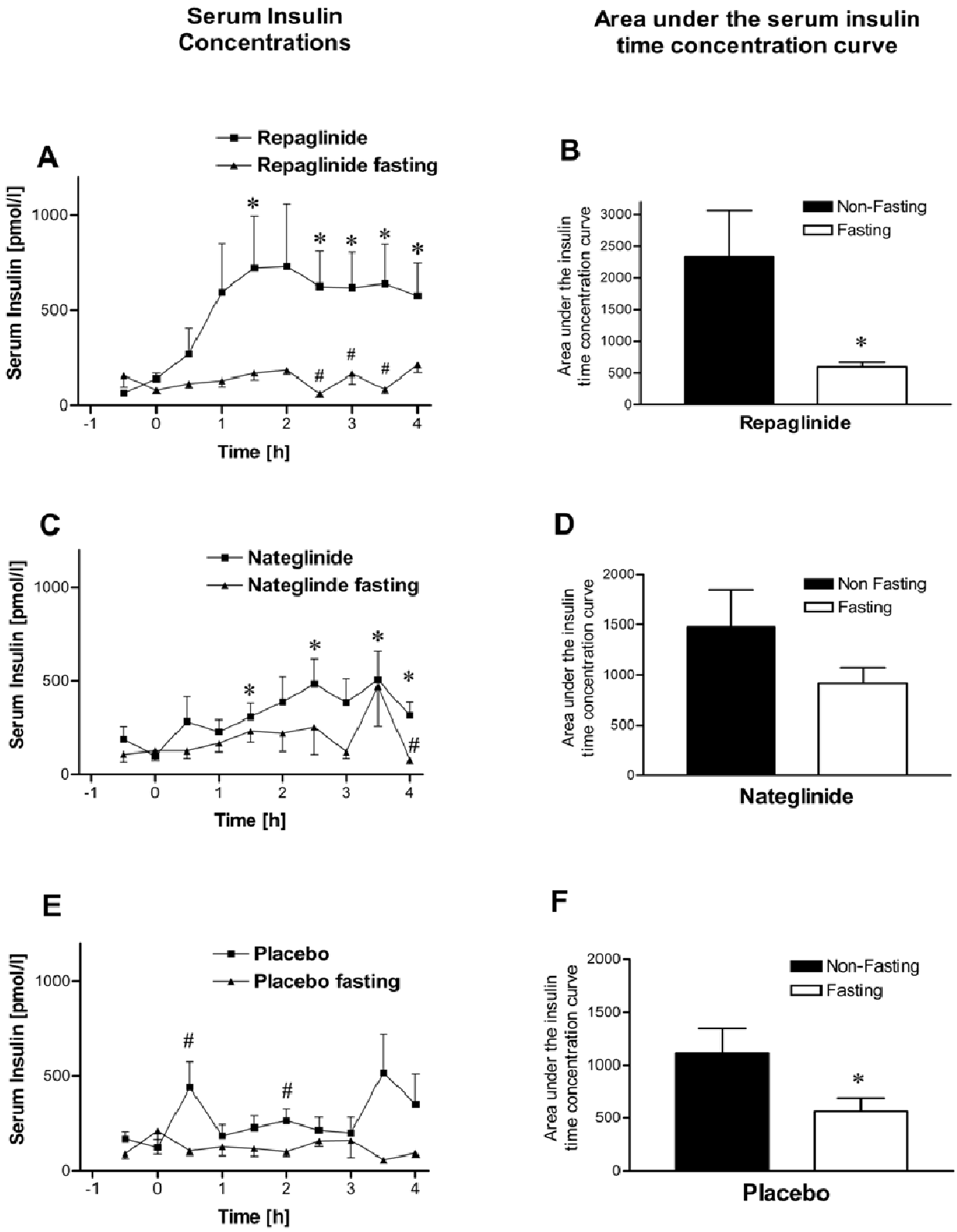

Figure 2. Mean serum insulin concentrations (± SEM) and the area under the time insulin concentration curve (AUCBL-4h) after treatments with 2 mg repaglinide (A&B), 120 mg nateglinide (C&D) and placebo (E&F) in the fasting and non-fasting state.

For serum insulin concentrations, *indicates $p < 0.05$; compared to baseline values; # indicates $p < 0.05$ compared to the corresponding time point of the fasting protocol. For area under the time insulin concentration curve, * indicates $p < 0.05$ compared to the non fasting state; paired Student's t test.

increased, if needed, to the maximum dose. On the other hand, in a pooled-analysis of four studies on Chinese patients, the rate of adverse reaction in nateglinide treated group for signs of hypoglycaemia was 2.11%, while that in repaglinide treated group was 1.05%. This was attributed to the use of a low dose of repaglinide (1 mg) and the maximum dose of nateglinide (120 mg) in one of the four studies (Li et al., 2009). The doses used in the present study were the maximum mealtime dose of nateglinide, and four times the starting dose of repaglinide. The maximum mealtime dose of repaglinide (4 mg) was not used because HbA1c of all patients was below 7.5%. Therefore using the 4 mg dose -8 times the starting dose- is not indicated. Further, repaglinide at the dose used, blunted the rise in serum glucose level, after breakfast, indicating that the dose used was adequate for the patients enrolled and no further increase in dose would have been required for these patients. Nateglinide, as well, blunted the rise in serum glucose level in the present study.

The modest rise in serum glucose level two hours postprandial in case of placebo administration may be due to the type of meal served, as both bran mixed bread and brown beans are low glycemic index foods. It has been shown that low glycemic index food induced a lower increment in the one hour posprandial plasma glucose levels (Wolever et al., 2008). Moreover, most of the enrolled patients were either newly diagnosed or controlled on metformin with HbA1c around 7%.

In the present study, both drugs showed a lower level of serum insulin level in the fasting state, though it was more pronounced and persistent in case of repaglinide. It is known that incretins, including glucagon like peptide (GLP-1) and glucose-dependent insulinotropic peptide (GIP), increase meal related insulin secretion. Nateglinide was shown to increase GLP-1 plasma level by inhibiting dipeptidyl peptidase IV (Duffy et al., 2007). Further, a recent study showed that nateglinide directly stimulates GLP-1 release by intestinal L cells *in vitro* as well as *in vivo* in the rat portal blood (Kitahara et al., 2011). Increasing insulin secretion through GLP-1 explains at least in part the mechanism, through which nateglinide does not release insulin as much in the fasting state. Repaglinide, on the other hand, did not affect plasma GLP-1 or GIP after oral glucose tolerance test in man (Stephens et al., 2011). Possible explanations for the glucose-dependent insulinotropic action of repaglinide are suggested to include altered counter regulatory hormone levels that are sufficient to inhibit insulin release in response to repaglinide in the fasting state (Aldhahi et al., 2004).

In light of the present results, the relatively low insulin levels after drug administration as compared to placebo, may be explained based on the composition of the meal served that resulted in a modest rise in plasma glucose level. In the present study, some of the enrolled patients had stable co-morbidities. None of these co-morbidities is a contraindication for the use of repaglinide or nateglinide. Meglitinides can be used in liver dysfunction, with the exception of severe cases (Inzucchi et al., 2012). Renal excretion of both drugs is minimal and they are considered an appropriate choice in individuals with more severe degrees of renal impairment (Del Prato et al., 2003; Hasslacher, 2003). As for ischemic heart disease, repaglinide appear to be associated with a lower cardiovascular risk than other insulin secretagogues (Schramm et al., 2011). Nateglinide was not among the insulin secretagogues studied. However several findings suggest a low cardiovascular risk with the use of nateglinide (Tentolouris et al., 2007).

Limitations

We used a generic formulation of nateglinide, since the reference listed drug (Starlix®) is not marketed in Egypt or Saudi Arabia at present. Therefore the authors decided to study the formulation available for clinical use for their patients. However, confirming the results concerning nateglinide in the fasting state using the reference formulation is warranted. Missing values is another limitation, however as mentioned above, they were substituted by EM method and were only 2.5% of the samples. The study was only performed in 8, almost treatment-naïve type II diabetic patients. However, as described earlier in the methods section, this rather small sample size was calculated based on the data provided by Rudovich et al. (2004). Enrolling more patients to our study would have not been approved by the ethical committee.

Conclusion

The present study suggests that in case of elderly who are at risk of missing meals, repaglinide may be a safe alternative to other anti-diabetics. Further studies are required, especially for nateglinide.

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