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Pentoxifylline Induces Lipolysis and Apoptosis of Human Preadipocytes, Keratinocytes and Fibroblasts *In Vitro*

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Abstract − Pentoxifylline (PTX) has been used for the local reduction of fat tissue in the clinical setting. However, its safety and efficacy have not been proven. The aim of this study was to evaluate the effects of PTX on cell lines established from fat tissue. Newly cultured human preadipocytes and adipocytes from subcutaneous abdominal fat in addition to purchased human lung fibroblasts and keratinocytes were treated with PTX at different concentrations. Cell viability was determined using the Cell counting kit (CCK)-8 assay and lipolysis was evaluated using an Elisa kit. DNA fragmentation, Western blot analysis, Hoechst and Propidium Iodide (PI) staining and fluorescence activated cell scanning analysis were performed to confirm apoptosis. The viability of adipocytes, preadipocytes, keratinocytes and fibroblasts was markedly decreased at concentrations of PTX above 20 mM. Apoptosis was induced at concentrations of PTX over 40 mM in all cell lines. Lipolysis was increased by 60% at concentrations of PTX of 20 mM compared to the control. In conclusion, the results of this study showed that 20 mM of PTX induced lipolysis. At concentrations over 20 mM, PTX reduced the viability of all cells studied including: adipocytes, preadipocytes, fibroblasts and keratinocytes, in a non-specific manner.

Keywords: Pentoxifylline, Apoptosis, Adipocyte, Preadipocyte, Fibroblast, Keratinocyte

INTRODUCTION

Adipose tissue mass is determined by competing processes regulating both the volume and number of adipocytes. With an increase in body weight, initially existing adipocytes become hypertrophied. As adipocytes exceed a critical cell size, the multipotent mesenchymal stem cells of adipose tissue are triggered to differentiate into mature adipocytes (Hauner *et al*., 1989; van Harmelen *et al*., 2003). The adipocyte size has been shown to correlate with the pattern of adipokine secretion; larger adipocytes have a more unfavorable profile of cytokine secretion than smaller adipocytes (Morin *et al*., 1998; Yang *et al*., 2004). Therefore, an optimal therapeutic approach to the treatment of obesity would regulate both the size and number of adipocytes.

Along with the growing national interest in obesity, a variety of treatment modalities have been developed; however, the safety and efficacy of most treatments have not been supported by scientific evidence. Mesotherapy, originally introduced in Europe, is a minimally invasive technique that consists of the intra- or subcutaneous injection of variable mixtures of natural plant extracts, homeopathic agents, pharmaceuticals, vitamins, and other bioactive substances (Accessed February 2nd, 2009). The use of mesotherapy in cosmetic medicine, to reduce and contour subcutaneous fat, is gaining popularity (Rotunda *et al*., 2005; Doerr, 2007), however, the safety and efficacy of this treatment has not been scientifically validated.

The compound 1-[5-oxohexyl]-3,7-dimethylxanthine (Pentoxifylline; PTX) is a common mesotherapy ingredient, used to increase blood circulation. As a non-specific phosphodiesterase (PDE)-inhibitor, PTX turns off tonic cyclic adenocine-3,5-monophophate (cAMP) hydrolysis, thereby contributing to a greater pool of cAMP and increased possible basal lipolysis (Snyder *et al*., 2005). Because of its hemorrhagic and anti-platelet properties, PTX has been used to treat peripheral vascular disease (Ward and Clissold, 1987; Moriau *et al*., 1995). PTX also has anti-

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oxidant activity as well as anti-TNF-α (Zabel *et al*., 1993) and anti-nuclear factor-κB (NF-κB) effects (Strieter *et al*., 1988). In a recent study carried out in a nonalcoholic steatohepatitis (NASH) mouse model, PTF administrated intra-abdominally significantly ameliorated NASH by inhibiting TNF-α and suppressing oxidative stress markers (Yalniz *et al*., 2007). Due to its anti-inflammatory, antioxidant and improved circulatory effects, PTF might improve the chronic inflammatory condition associated with obesity.

Adult human white adipose tissue has been reported to be composed of approximately 50-70% adipocytes, 20-40% stromal vascular cells (*i.e.* preadipocytes, fibroblasts, multipotent mesenchymal cells), and 1-30% of infiltrated macrophages (Hauner, 2005). Therefore, the effects of PTX must be studied on other cell lines in addition to adipocytes. This is the first study to investigate the effects of PTX on human adipose tissue. The goal of this study was to investigate the effects of PTX on human adipocytes, preadipocytes, fibroblasts and keratinocytes *in vitro* to determine whether PTX induces lipolysis and apoptosis of adipocytes and preadipocytes, and determine whether PTX is specific for adipocytes and preadipocytes.

MATERIALS AND METHODS

Specimens

Adipose tissue samples were isolated from lipoaspirates obtained during elective liposuction surgery. Samples were collected from patients with no significant medical history and were not taking any medication. Approval for this study was obtained from the institutional review boards (IRB) of the Catholic University of Korea, College of Medicine and informed consent was obtained according to the Declaration of Helsinki.

Isolation and culture of preadipocytes, keratinocytes and fibroblasts

The preadipocytes were prepared by isolating them from lipoaspirates in the following manner: the lipoaspirates were washed three times with phosphate-buffered saline (PBS, Gibco-BRL, Grand Island, N.Y., USA) containing 10% antibiotic/antimycotic (P/S, Gibco-BRL) and the extracellular matrix was digested with 0.06% collagenase (collagenase type I, Invitrogen Corporation, Carlsbad, CA, USA) and 1% bovine serum albumin (BSA, Bovogen, Ogilvie St. Essendon Vic, Australia). The above solution with the lipoaspirates were then placed in a shaking water bath for 2 hours at 37°C, followed by filtration through a 40 μM nylon mesh (cell strainer, BD Falcon, Two oak park Bedford MA, USA). Using erythrocyte lysis buffer (0.15 M ammonium chloride, 1.0 mM potassium bicarbonate and 0.1 mM EDTA), the erythrocytes were removed and the remaining cells were seeded in culture flasks while they were maintained under conditions identical to the conditions used for the preadipocytes. The cells were cultured in Minimum Essential Medium α (α-MEM, Gibco-BRL) supplemented with 10% Fetal bovine serum (FBS, Hyclone, Logan, Utah, USA) and 1% Penicillin- Streptomycin (P/S, Gibco-BRL) and left in a humidified atmosphere containing 5% CO_2 at 37°C. After 24 hours, the cultured cells were washed with PBS to remove non-adherent material. The medium was replaced twice a week during the third passage (P3). The keratinocyte and fibroblast cells were purchased from the American Type Culture Collection (ATCC, Manassas VA, USA). These cells were cultured in Dulbeco`s modified Eagles 1s media (DMEM, Gibco-BRL) supplemented with 10% FBS (Hyclone) and 1% P/S (Gibco-BRL), and maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell survival assay

Adipocytes: The preadipocytes were plated at 15,000 cells/well in 96-well plates and incubated for 24 hours. These cells were then treated with adipogenic media (DM-2/10, Zen-Bio, Inc., Research Triangle Park, Bovard, NC.,USA). After 14 days, the cells were further treated with 0, 20, 40, 60, 80, and 100 mM of PTX. After 24, 48, and 72 hours, the cells were treated with a 1/10 volume of CCK-8 (Dojindo Molecular Technologies, Rockville, MD USA). After 2 hours of treatment with CCK-8, the cells were assessed for viability using an ELISA reader.

Other cells

Starvation: The preadipocytes, fibroblasts and keratinocytes were seeded 1.5×10^4 cells/well in 96-well plates. After 24 hours, the cells were exchanged with 10% FBS media. After 3 days, these cells were treated with 0, 20, 40, 60, 80, and 100 mM of PTX. After 24, 48, and 72 hours, the cells were assessed for viability using an ELISA reader as described for the adipocytes.

Without starvation: The preadipocytes, fibroblasts and keratinocytes were seeded 5×10^4 cells/well in 96-well plates. After 24 hours, these cells were treated with 0, 20, 40, 60, 80, and 100 mM of PTX. After 24, 48, and 72 hours, they were assessed for viability using an ELISA reader as described for the adipocytes.

Lipolysis assay

The preadipocytes from the third passage were plated

onto 96 well plates with 5×10^4 cells/well and cultured for 24 hours in control medium (DMEM with 10% FBS and 1% antibiotic/antimycotic at 37 and 5% CO_2); then the medium was replaced with adipogenic medium (DM-2/10, Zen-Bio, Inc., Research Triangle Park, Bovard, NC.,USA). The culture medium was changed every 3 days and cultured for 2 weeks.

After treatment with different concentrations of pentoxifylline (0, 2 nM, 20 nM, 200 nM, 2 mcM, 2 mM, 20 mM) added to the culture medium, newly differentiated adipocytes were analyzed by a lipolysis assay according to the method of Brasaemle (Brasaemle, Dolios *et al*., 2004).

DNA fragmentation

The preadipocytes, fibroblasts and keratinocytes were plated at 9×10^5 cells/ well in 60 mm dishes and incubated for 24 hours. The cells were treated with 0, 20, 40, 60, 80, and 100 mM of PTX. After 24 hours, DNA was extracted using the Qiagen mini kit (Qiagen; Max-Volmer-Strabe, Hilden, Germany). Electrophoresis (using 1% agarose gel with ethidium bromide) of 5 μg of DNA from each cell line was separated for further analysis. The changes were evaluated under a UV detector.

Nuclear condensation:

The preadipocytes, fibroblasts and keratinocytes were plated at 9×10^5 cells/ well in 60 mm dishes and incubated for 24 hours. The cells were treated with 0, 20, and 40 mM of PTX for 24 hours. Then 3.7% formaline was added for 10 minutes and 4',6- Diamidino-2-phenylindole, dihydrochloride (Sigma) and Propidium Iodide (PI) was added at 5 ug/well. The nuclei were stained with Hoechst 33258 and the changes of the nuclei were evaluated under a florescent microscope.

Western blot analysis

Antibodies for Bcl-2, Bax, Caspase 3 and GAPDH were purchased from R&D Systems (R&D Systems, Minneapolis, MN., USA). The preadipocytes, fibroblasts and keratinocytes were plated at 9×10^5 cells/ well in 60 mm dishes and incubated for 24 hours. The cells were treated with 0, 20, 40, 60, 80, and 100 mM of PTX. After 24 hours, the cells were lysed in RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 lM Na3VO4, 1 mM DTT, and 50 lg/ml PMSF) for 1 hour on ice. The insoluble materials were removed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was then directly subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE). The proteins were transferred to a nitrocellulose membrane. The blots were blocked in phosphate-buffered saline containing 5% skim milk and 0.05% Tween 20. This was followed by incubation with the primary and HRP-conjugated secondary antibodies. Detection was performed according to the enhanced chemiluminescence protocol (Amersham, Arlington, Heights, IL., USA).

Statistical analysis

Cell viabilities and lipolysis were expressed as mean ± standard deviation. The significance level of treatment effects was determined using one way analysis of variance (ANOVA) followed by Tukey's post- hoc analysis and *p*-values lower than 0.05 was considered statistically significancy.

RESULTS

The culture of isolated preadipocytes reached confluence on day 3. Adding adipogenic differentiation medium resulted in the preadipocyte cells increasing in size, becoming rounder and the presence of intracellular lipid droplets in the cytoplasm. The cell population on day 14 was approximately 50% adipocytes containing small lipid droplets; the adipocytes were stained with oil red-O on day 21. The pH of the culture medium ranged from 6.7 at 20 mM of PTX to 7.1 at 80 mM PTX (Table I).

Effect of PTX on cell viability

After 24 hours of incubation with PTX, adipocyte viability was decreased by 58.3% at 20 mM and a similarly reduced viability was detected above 40 mM of PTX (Fig. 1A). After 48 and 72 hours of incubation, the adipocyte viability showed similar results. The viability of preadipocytes, keratinocytes and fibroblasts decreased significantly at concentrations of PTX above 20 mM (Fig. 1B-D respectively). The cell viability was decreased more with starvation than with 10% FBS added to all three cell lines.

Effect of PTX at lower concentrations on adipocyte viability and lipolysis

Adipocyte viability was slightly decreased, by 20%, with 2 mM of PTX (Fig. 2A). From 2 nM to 2 mM of PTX, lipolysis increased by 10% compared to the control, and it increased by 60% at 20 mM of PTX (Fig. 2B).

Table I. pH changes of dissolved media according to various pentoxifylline (PTX) concentration

Concentration (mM)	0	20	40	60	80	100
p H	6.8	6.7	6.9	7.0	7.1	7.0

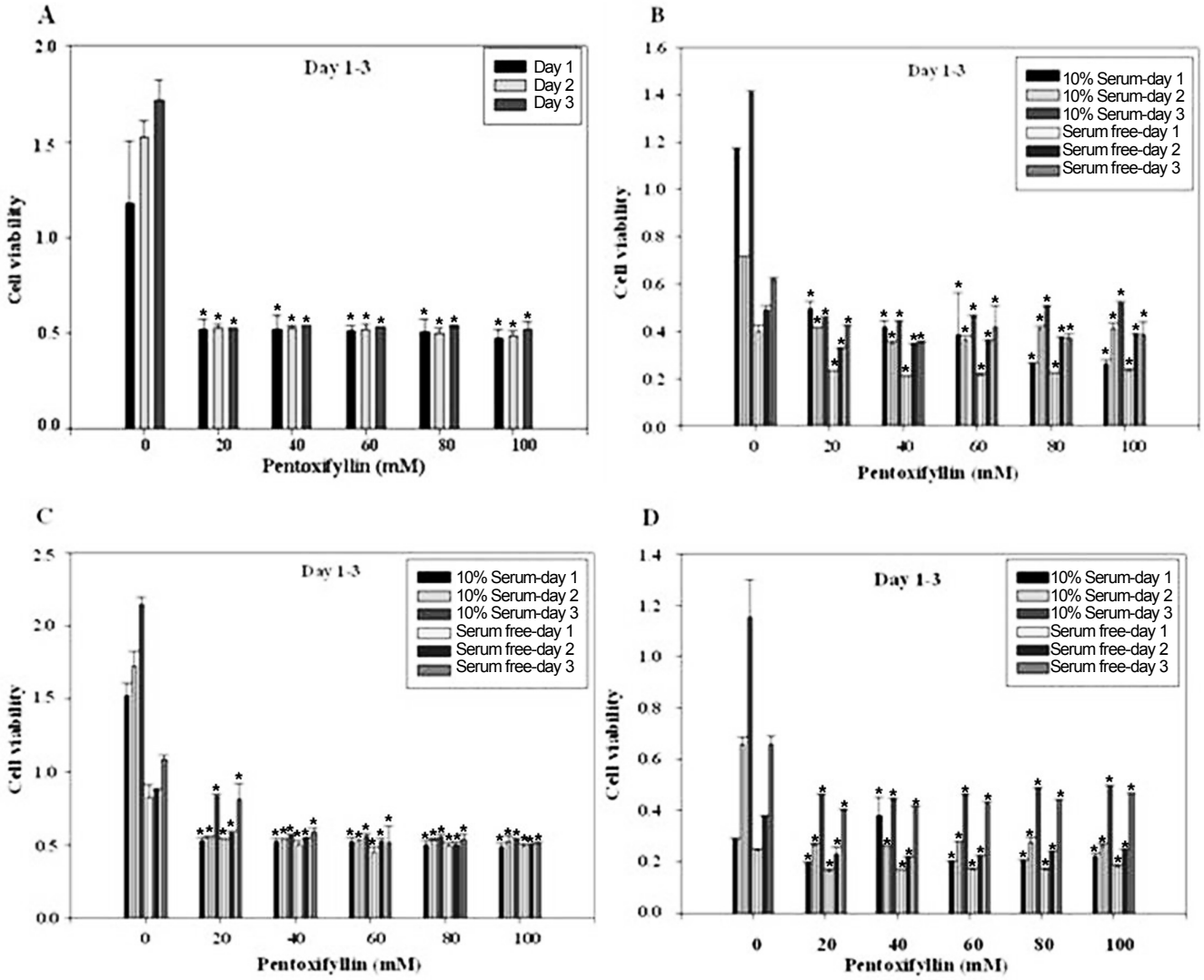

Fig. 1. The effect of pentoxifylline (PTX) on human adipocyte (A), human preadipocyte (B), human keratinocyte (C) and human lung fibroblast (D) cell viability. After 24 hours of incubation with PTX, adipocyte viability was decreased significantly at concentrations above 20 mM PTX. The viability of preadipocyte, fibroblast and keratinocyte cells was decreased at concentrations above 20 mM in both the starvation and 10% FBS groups. The numbers in the left column represent relative viability. The standard deviations are shown on top of each bar. These experiments were performed at least three times in duplicate. *$p < 0.05$ by Tukey's post-hoc analysis.

Effect of PTX on preadipocyte, keratinocyte and fibroblast cell Apoptosis

DNA fragmentation was not observed in the control samples and at 20 mM of PTX; however, it was detected above 40 mM of PTX (Fig. 3). Nuclear condensation was observed at 20 mM of PTX and complete condensation was observed at 40 mM of PTX (Fig. 4). The expression of Bcl-2, did not differ at different concentrations of PTX in the preadipocyte, fibroblast and keratinocyte cells; however, the expression of Bax and Caspase-3 showed a dose-response correlation in up to 60 mM of PTX. Above 60 mM of PTX, the expression of Bax and Caspase-3 decreased (Fig. 5).

DISCUSSION

Recent studies have consistently shown hypoxic responses of adipose tissue (Kabon *et al*., 2004; Fleischmann *et al*., 2005; Rausch *et al*., 2008). This finding has suggested that cellular metabolism is associated with obesity (Hosogai *et al*., 2007; Ye, 2009). In obese individuals, the adipose tissue blood flow rate has been shown to be 30-40% lower than in non-obese subjects (Bolinder *et al*., 2000).

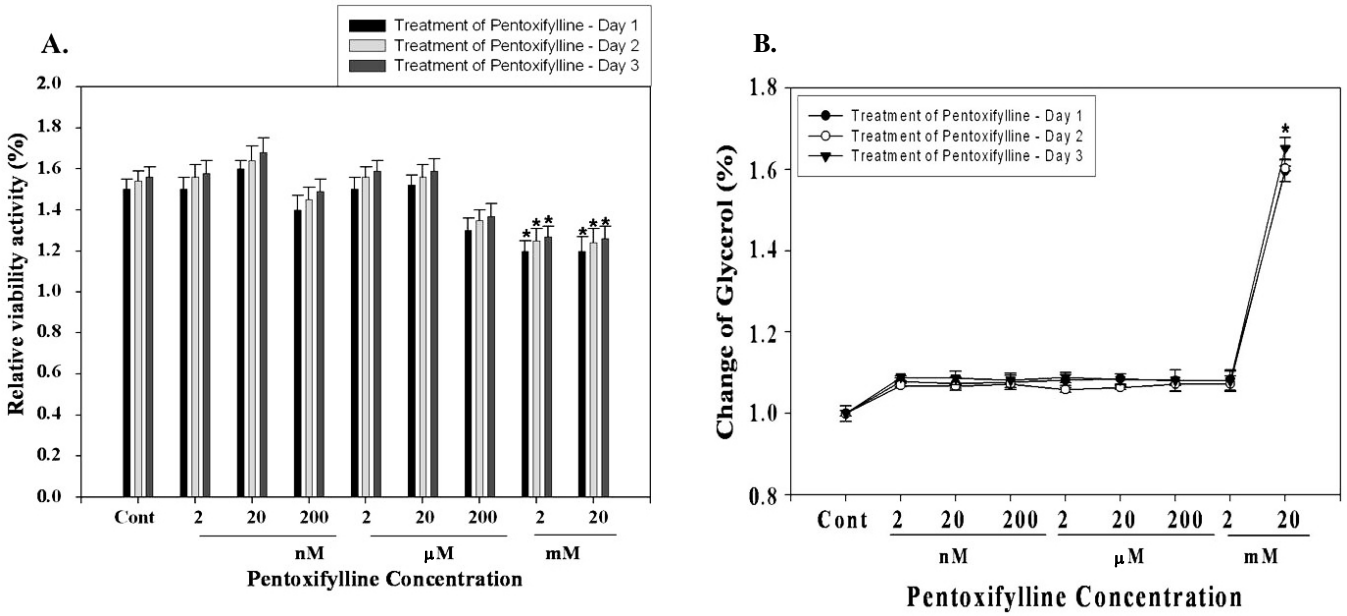

Fig. 2. The effect of pentoxifylline (PTX) at lower concentration on human adipocyte viability (A) and lipolysis (B). Adipocyte viability was slightly decreased above 2 mM of PTX. Lipolysis was increased by 10% at concentrations from 2 nM to 2 mM of PTX and by 60% at a concentration of 20 mM of PTX. The numbers in the left column represent relative viability and change of the glycerol level. The standard deviations are shown on the top of each bar. These experiments were performed at least three times in duplicate. *$p < 0.05$ by Tukey's post-hoc analysis.

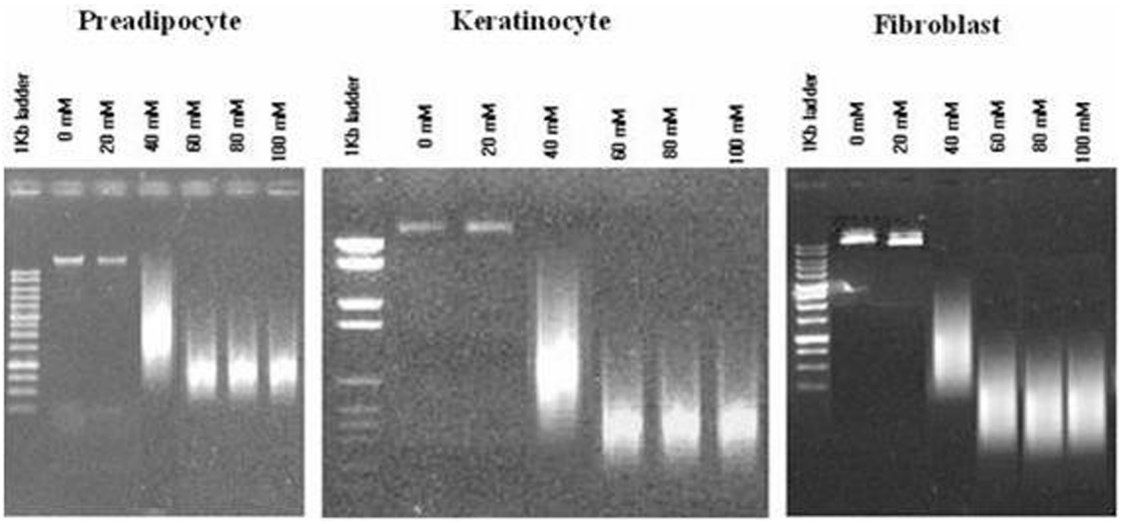

Fig. 3. The effect of pentoxifylline (PTX) on the DNA fragmentation of each cell line. DNA fragmentation was not observed in the controls and at 20 mM of PTX; it was detected above 40 mM of PTX. DNA was extracted from each of the cell lines and gel electrophoresis performed after the indicated treatment concentrations of PTX for 24 hours. Lane 1 (M): 1 Kb size marker, Lane 2 through 7: treatment of 0, 20, 40, 60, 80, and 100 mM concentration of PTX.

PTX improves the circulation, primarily by increasing red blood cell deformability, by reducing blood viscosity and by decreasing the potential for platelet aggregation and thrombus formation (Ward and Clissold, 1987; Moriau *et al*., 1995). PTF also inhibits proinflammatory cytokines. PTX blocks NF-κB activation and suppresses NF-κB dependent synthesis and the release of TNF-α (Strieter *et al*., 1988; Zabel *et al*., 1993). Therefore, PTX may serve as a

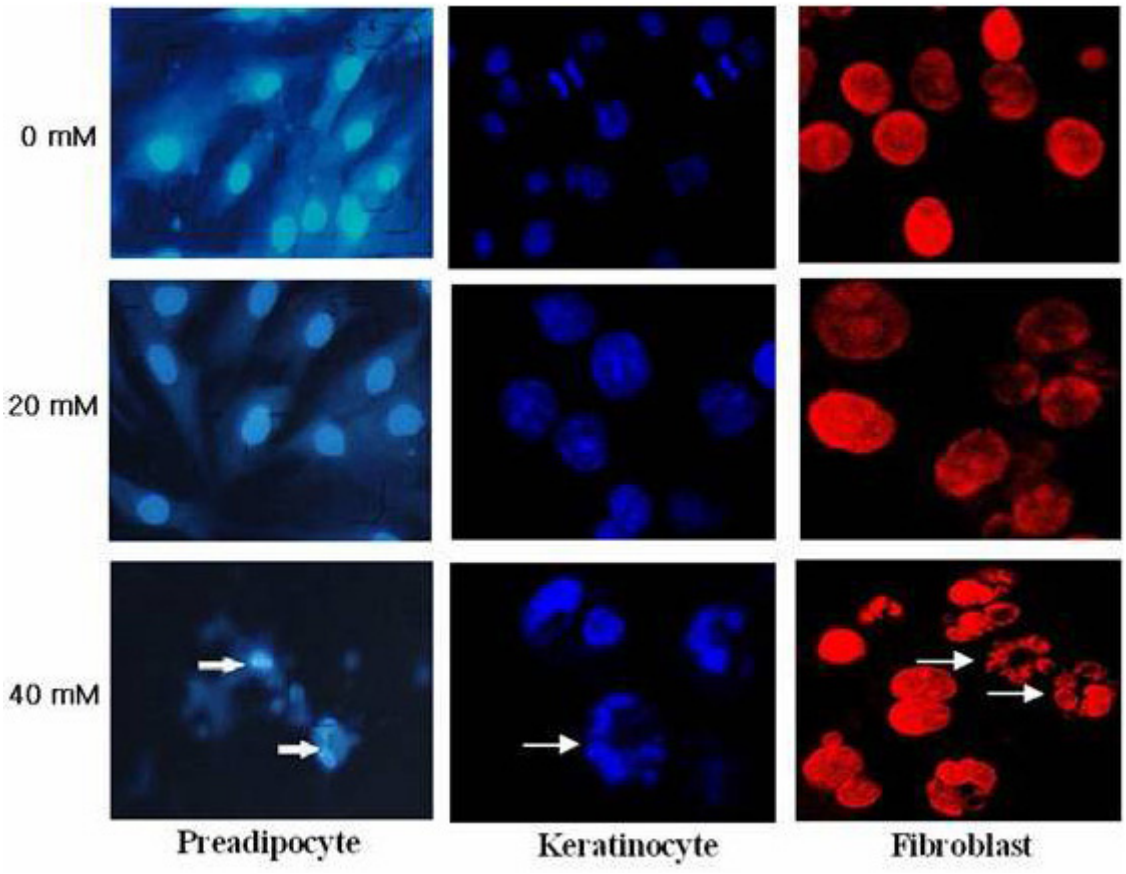

Fig. 4. The effect of pentoxifylline (PTX) on nuclear condensation for each cell line. The cells were treated with the indicated concentration of PTX for 24 hours. Then, 3.7% formaline was added for 10 minutes and 4',6-Diamidino-2-phenylindole, dihydrochloride and Propidium Iodide (PI) injected at a dose of 5 μg/well. The nuclei were stained with Hoechst 33258 and the changes in the nuclei were evaluated under a florescent microscope. DNA is shown in the blue and red colors. Nuclear condensation was detected at concentrations above 20 mM PTX and complete condensation was observed at 40 mM of PTX.

potential adjunct therapeutic agent for the treatment of conditions where TNF-α production plays a significant role. PTX has been reported to be a possible agent for the treatment of steatohepatitis (Yalniz *et al*., 2007), sepsis (Selim *et al*., 2004), kidney disease (Ducloux *et al*., 2001), as well as cancer (Rauko P *et al*., 1998; Rishi *et al*., 2009). In the NASH animal model, PTX significantly ameliorated the histopathological lesions associated with NASH and decreased the aminotransferase levels by its antioxidative and anti-TNF-α effects (Yalniz *et al*., 2007). Prior to these findings, the only clinically confirmed treatment of NASH was weight reduction (Moschen and Tilg, 2008).

Fat dissolving local injections have two therapeutic effects; First, the injected reagents are toxic to adipose and other associated cells, causing permanent removal of fat tissue; second, the injections have been associated with a temporary decrease in the fat stores, resulting in smaller fat cells (Rotunda *et al*., 2005). The results of this study showed that the effects of PTX on adipocyte cells differed according to the PTX concentration. PTX concentrations above 20 mM reduced adipocyte viability; at lower concentrations (2 nM-20 mM), PTX induced lipolysis, which was 60% at 20 mM concentrations. The effects of PTX on preadipocyte, fibroblast and keratinocyte cells were similar, above 20 mM of PTX; the viability of all three cell lines was decreased and the expression of Bax and Caspase-3 showed a dose-response correlation in up to 60 mM of PTX. Even though there is no comparable report of PTX induced apoptosis, decreased expression of of Bax and Caspase-3 above 60 mM of PTX might be explained by posttranslational proteins degradation at high concentration of PTX.

Therefore, the effects of PTX above 20 mM were not cell specific. Previous studies have reported that 20 mM PTX was the minimum concentration necessary to produce a reproducible and reliable down-regulation of TNF-α in lip-

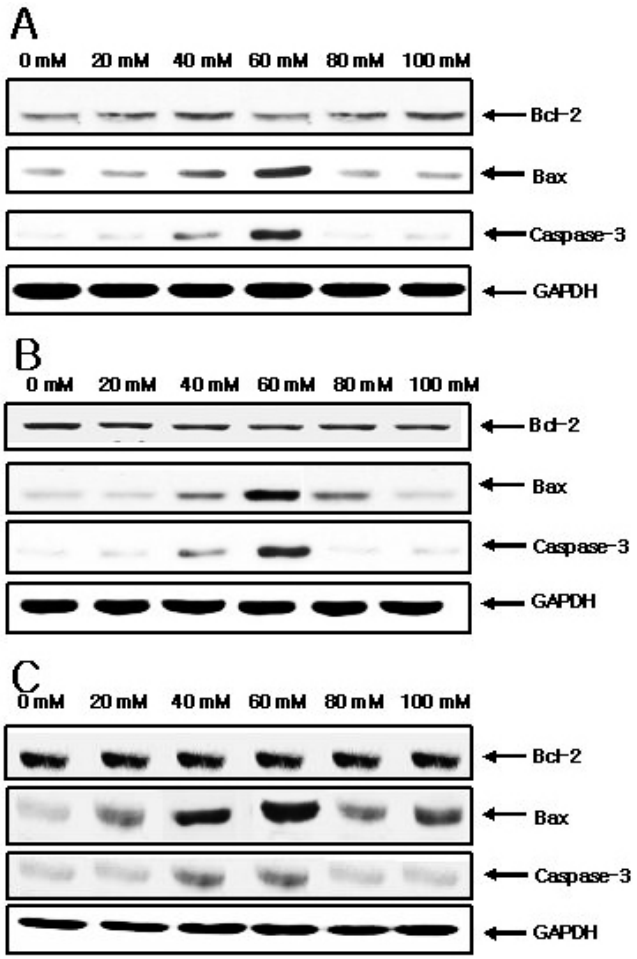

Fig. 5. The effect of pentoxifylline (PTX) on Bcl-2, Bax, and Caspase-3 expression in preadipocyte (A), keratinocyte (B) and fibroblast (C) cells. The cells were treated with 0, 20, 40, 60, 80, and 100 mM of PTX for 24 hours and were lysed in RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 lM Na3VO4, 1 mM DTT, and 50 lg/ml PMSF) for 1 hour. The supernatant was subjected to SDS-PAGE. The blots were blocked in phosphate-buffered saline containing 5% skim milk and 0.05% Tween 20, followed by incubation with the primary and HRP-conjugated secondary antibodies. Detection was performed according to the enhanced chemiluminescence protocol. The expression of Bcl-2 did not differ at different concentrations of PTX; however, the expression of Bax and Caspase-3 had a dose-response correlation up to 60 mM of PTX. The expression of Bax and Caspase-3 was decreased at concentrations above 60 mM PTX.

opolysaccharide stimulated peripheral blood mononuclear cells (Coimbra *et al*., 2005; Deree *et al*., 2008). The currently available PTX concentration in Korea is 73 mM. However, there are a variety of different formulas used for mesotherapy. Therefore, although we investigated a wide range of PTX concentrations, we do not know if the doses studied correspond to those used clinically for any given patient.

The results of our study showed that PTX below 20 mM induced lypolysis in adipocytes; there are several possible underlying mechanisms that might explain this. As a non specific phosphodiesterase inhibitor, PTX turns off tonic cAMP hydrolysis, contributing to a greater pool of cAMP and increased possible basal lipolysis (Schandené L *et al*., 1992). Another possibility is related to the findings that TNF-α increases the rate of lipolysis in humans *in vivo* (Starnes *et al*., 1988) and in primary cultures of newly differentiated human preadipocytes (Hauner *et al*., 1995). If PTX below 20 mM cannot block TNF-α completely, TNF-α might induce lypolysis.

The effects of PTX induced non specific apoptosis in all three cell lines studied; this result could be explained by the following. PTX has been reported as a potential chemotherapeutic agent. NF-κB regulates the expression of many genes that are critical for apoptosis (Rishi *et al*., 2009). In addition, PTX might be associated with non-specific cell stress that leads to apoptosis. However, it is unclear how TNF-α would cause apoptosis. Prins *et al*. found that 425 ng/ml of TNF-α induced apoptosis of human white adipocyte cells *in vitro* (Prins *et al*., 1994). Because this level of TNF is too high to be considered within the normal physiologic range, it may have relevance only for extreme conditions such as cachexia or sepsis (Qian *et al*., 2001).

In conclusion, the results of this study showed that PTX at 20 mM induced lipolysis and that concentrations above this level reduced the cell viability of adipocytes, preadipocytes, fibroblasts, and keratinocytes in a non specific manner. Further studies including *in vivo* studies are now needed to further explore the effects of PTX.

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