권미정 석사학위논문

Hepatic differentiation of human adipose tissue-derived mesenchymal stem cells and adverse effects of arsanilic acid and acetaminophen during in vitro hepatic developmental stage

인체 지방유래 중간엽 줄기세포의 간세포 분화 및 비소와 아세트아미노펜의 간세포 분화에 미치는 영향 평가

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지도교수 조 제 열

이 논문을 권 미 정 석사학위논문으로 제출함

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권 미 정의 석사학위논문을 인준함

2016년 월

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Abstract

In the present study, the hepatocyte-like cells (HLCs) were differentiated from human adipose tissue-derived mesenchymal stem cells (AT-MSCs). The hepatic differentiation was confirmed by increases in hepatic proteins or genes, the cytochrome P450 (CYP) activities, albumin secretion and glycogen storage. To determine the developmental toxic effect of Arsanilic acid (Ars) and acetaminophen (AAP) on the hepatic development, the differentiating cells were treated with the test chemicals (below IC12.5)from day 4 to day 13. The enzymatic activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) did not significantly differ in response to Ars treatment. AAP treatment increased the activities of all enzymes in a dose-dependent manner, significantly at concentrations of 2.5 and 5mM AAP. On the expressions of hepatic genes for Ars, the expressions were significantly inhibited by more than 0.5 mM for Albumin (ALB) but only 2.5 mM for α-feto protein (AFP). In the AAP-treated group, the expressions of ALB and AFP were significantly decreased at the concentrations exceeding 0.625 mM. The activities of CYP3A4 were not changed by both treatments. The activities of CYP1A2 were increased by AAP, whereas it was decreased by Ars treatment. In conclusion, AAP could cause serious adverse effects during the hepatic development as compared to Ars.

Keywords: Human adipose tissue-derived mesenchymal stem cell, Hepatocyte-like cell, Hepatotoxicity, Arsanilic Acid, Acetaminophen

Student number: 2010-21637

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Fig. 4. MTT assay for non-cytotoxic concentrations of chemicals. After treatment of non-cytotoxic concentrations for 10 days of hepatic differentiation, 1 and 2.5 mM Ars (a) and 5 mM AAP (b) were cytotoxic to HLCs compared to the non-treated control group. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

Fig. 5. Enzymatic effects after the treatment with Ars and AAP. Cellular damage was estimated by LDH, ALT and AST after treatment with Ars (a) and AAP (b) for 10 days of hepatic differentiation. Enzyme activities were expressed as Unit per Liter (U/L) and the values were normalized by protein concentration (mg/ml). Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

Fig. 6. Expressions of hepatocyte-specific genes after treatment with Ars and AAP. The expressions of ALB and AFP were evaluated in response to Ars (a and b) and AAP treatment (c and d). Data are expressed as the fold increase in the mRNA level compared to the non-treated group. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

Fig. 7. CYP activities after treatment with Ars and AAP. CYP1A2 significantly decreased in a dose-dependent manner in response to Ars (a) while it significantly increased in response to AAP (c). The CYP3A4 activities did not significantly differ between both chemicals (b and d). Data are expressed as the fold increase compared to the non-treated group. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

Introduction

The liver is a target organ for drug-induced toxicity and plays a key role in metabolising xenobiotics via enzymes, such as cytochrome P450 (CYP). The side effects of candidate drugs on the liver have been a pressing issue for human safety and the drug industry and represent the most frequent cases for the withdrawal of an approved drug after marketing. Thus, the evaluation of liver toxicity is important in drug development. Traditionally, the toxicity of a given chemical has been tested using an animal model. Previous reports showed that the hepatotoxic effects of some pharmaceuticals were not distinguishable from those of others in an animal model (Olson et al, 2000;Olson et al, 1998). The use of human hepatocytes would overcome the disadvantages of animal models and may identify potential liver toxicity in humans. However, hepatocytes have a limited capacity for proliferation and lose their hepatic traits after prolonged culture. The scarcity of donor organs presents an obstacle for obtaining primary hepatocytes. Currently, immortalised cell lines, including HepG2, have been used as alterative models. However, immortalised cells have an inherent disadvantage, such as poor liver function and instability in culture (Greenhough et al, 2010;Wong et al, 2000). Therefore, a reliable model to evaluate hepatotoxicity is needed in order to identify potential toxicity in preclinical studies. (Al Battah et al, 2011;Brandon et al, 2003).

Together with advanced stem cell biotechnology, adult stem

cells have been considered a useful source for in vitro models of toxicological studies. Many studies have been focused on directly differentiating mesenchymal stem cells (MSC) originated from the bone marrow and adipose tissue into hepatocyte-like cells (HLC) (Okura et al, 2010a;Pournasr et al, 2011). Adipose tissue-derived mesenchymal stem cells (AT-MSCs) have potential benefits, such as proliferation ability, plasticity to generate other cell types and a more readily available source of human cells (Lee et al, 2004;Musina et al, 2005;Zhu et al, 2008). The biological potency of AT-MSCs is similar to that of bone marrow-derived MSC (BM-MSCs) (Izadpanah et al, 2006;Katz et al, 2005;Kern et al, 2006), and AT-MSCs are more abundant than BM-MSCs (Strem et al, 2005).

Arsenic compounds have been used as a drug, agricultural pesticide and feed additives, but arsenic is now classified as a group I human carcinogen in the IARC (IARC, 2012). Arsanilic Acid (Ars) is an organic arsenical, which is considered less toxic than inorganic arsenic compounds. Ars has also been used as a feed additive in the poultry and pig industry (Parker and Walker 1976;VanderKop and MacNeil 1989). Recently, Ars was reported to induce apoptosis and oxidative stress in rats (Lu et al, 2014;Zhang et al, 2011). Acetaminophen (AAP) is a widely used antipyretic analgesic, but AAP overdose causes hepatotoxicity in humans and animals (James et al, 2003). Liver injury derived from AAP overdose remains a serious public health problem. AAP overdose is the leading cause of acute liver failure (Ben-Shachar et al, 2012;Lee 2008;Nourjah et al, 2006).

In the present study, human AT-MSCs were differentiated to HLCs and the MSC-derived hepatic differentiation was used as in vitro alternative model for investigating the adverse effects of AAP and Ars on the hepatic developmental stages.

Materials and Methods

Isolation of AT-MSCs

Abdominal subcutaneous and breast adipose tissue were obtained by surgical method after acquiring the written informed consent form patients, in accordance with the guidelines of Samsung Medical Center Institutional Review Board (IRB number; SMC 2011-08-111-002). Human AT-MSCs were harvested from the adipose tissues of male and female patients between the ages of 27 and 53. Participating patients had no prior knowledge or evidence of ongoing systemic disease at the time of operation. Isolation of AT-MSCs proceeded as previously described (Boquest et al, 2006).

Briefly, adipose tissues were washed at least three times with sterile Hank's Balanced Salt Solution (HBSS, Gibco-BRL, Paisley, UK). The adipose tissue minced with scissors was treated with equal volume of 0.2% Collagenase type I (Sigma-Aldrich, St Louis, MO, USA) solution for 1 h at 37°C with intermittent shaking and then cells were separated by centrifugation at 400xg for 5 min after addition with 10% Fetal Bovine Serum (FBS, Gibco-BRL, NY, USA). The pellet was resuspended with 1x RBC lysing buffer (eBioscience, GA, USA) and placed for 10 min at room temperature (RT). After centrifugation at 300xg for 5 min, the pellet was resuspended with HBSS supplemented with 2% FBS and 1% penicillin/streptomycin (Gibco-BRL). The suspension was passed through 100 μm and 40 μm cell strainer (BD Falcon, NJ, USA) to remove cell clumps. The floating

cells were separated by centrifugation at 300xg for 5 min. The isolated cells were cultured with Mesenchymal Stem Cell Basal Medium (ATCC, USA) including mesenchymal stem cell supplement and L-Alanyl-L-Glutamine (ATCC) in culture plate coated with 0.1% gelatin.

For obtaining the purified MSCs from the pooled primary cells, AT-MSCs were collected by magnetic activated cell sorting (MACS) with CD105 microbeads and MACS LD columns (Miltenyibiotec, CA, USA).

Hepatic differentiation procedure

The strategy of hepatic differentiation is illustrated in Fig. 1. I modified the hepatic differentiation protocol by Banas et al (Banas et al, 2007). AT-MSCs (passage 3 or 4) were plated at a density of 8 x 10^4 cells/ml on Collagentype I-coated plate (IWAKI, Japan) in Mesenchymal Stem Cell Basal Medium. At 90 to 100% confluence of AT-MSCs, the medium was removed and replaced with endodermal induction medium for 3 days (Step-1). The endoderm induction medium consists of DMEM supplemented with Activin A (20ng/ml, Sigma-Aldrich) and fibroblast growth factor 4 (FGF4;20ng/ml, Sigma-Aldrich). Afterward, the definitive endodermal cells were differentiated into HLCs by hepatic differentiation medium for 10 days (Step-2). The hepatic differentiation medium consists of DMEM supplemented with hydrocortisone (1μM,Sigma-Aldrich), bovine serum albumin (BSA;0.5mg/ml, Sigma-Aldrich), ascorbicacid (0.65mM, Sigma

-Aldrich), epidermal growth factor (EGF;20ng/ml, Sigma-Aldrich), hepatocyte growth factor (HGF;150ng/ml, Sigma-Aldrich), fibroblast growth factor1 (FGF1;100ng/ml, Sigma-Aldrich), FGF4 (25ng/ml, Sigma-Aldrich), oncostatin M(30ng/ml,Sigma-Aldrich), dexamethasone (20μM,Sigma-Aldrich), insulin-transferrin-selenium(ITS;Sigma-Aldrich) and nicotinamide (50μM, Sigma-Aldrich).

Chemicals treatment

Ars and AAP were purchased from the Sigma-Aldrich. The chemicals were initially dissolved as concentrated stock solution in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted with medium. Non-treated control group was incubated with medium including same final concentration of 0.1% DMSO.

Immunocytochemical assay

For immunoflouorescence analysis, the cells were fixed with 4% paraformaldehyde (PFA, Thermo Science, USA) for 10 min after washing with Phosphate Buffered Saline (PBS). After rinsing with 1x rinse buffer (1X Tris-HCl including 0.05% Tween-20), permeabilization was conducted with 0.1% Triton X-100 for 10 min. After blocking with Blocking Solution (1:20 diluent with 1x PBS) for 30 min at RT, the plates were incubated overnight at 4°C with anti-α-feto protein (AFP; Santa cruz Biotechnology, CA, USA), anti-cytokeratin 18 (CK18; Santa cruz Biotechnology, CA, USA) and anti-albumin (ALB; Abcam, MA, USA). After rinsing, the Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen, CA, USA) for AFP,

Cy3-conjugated goat anti-mouse IgM/IgG (Stemgent, USA) for ALB and Cy3-conjugated goat anti-rabbit IgG (Stemgent) for CK18 was applied to incubate for 2 h at RT. Nuclei were stained for 1 min with Hoechst 33258 (Invitrogen) diluted in 1x PBS (1:10000). Immunofluorescence was detected under a fluorescence microscope (Axiovert, Carl Zeiss, NY, USA)

RT-PCR

RNA was extracted from cells using the Easy-spin Total RNA Extraction kit (iNtRON Biotechnology, INC, USA). cDNA synthesis was performed using 1 μg of total RNA with AccuPower RT PreMix (Bioneer, Seoul, Republic of Korea). PCR cycling conditions consisted of denaturation at 95°C for 10 sec, annealing at 45°C to 64°C for 10 sec, extension at 72°C for 20 sec for up to 45 cycles. Real-time PCR was carried out by using the iCycler iQ Real-Time PCR Detection System. Gene-specific expression values were normalized to level of GAPDH within each sample and relative to control value.

PAS staining

To detect glycogen storage, PAS staining was performed from the Sigma-Aldrich. Cells were fixed for 2 min with 4% PFA and oxidized for 5 min in periodic acid at RT. After rinse with distilled water for 5 min, the cells were treated with Schiff's reagent for 15 min.

CYP enzyme activity

CYP enzyme activities were assessed using CYP450-Glo™ assay kit (Promega, WI, USA) specific for CYP1A2 and CYP3A4 according to manufacturer's instruction. AT-MSCs (4 x 10^5 cells/well) were differentiated to HLCs in Collagentype I-coated 24-well plate. After treatment of reagent during hepatic induction, the supernatant of each well was transferred to white opaque 96-well plate and then CYP activities were measured by Luminometer (Flexstation III, Molecular Device, USA). The result was compared with levels in undifferentiated AT-MSCs and HLCs at day 13 and expressed as fold change against AT-MSCs.

Albumin secretion

Albumin amounts were estimated using Albumin (Human) ELISA kit (Abnova, USA) according to manufacturer's instruction. Briefly, the supernatants were collected from AT-MSCs, HLCs and HepG2 after 24h incubation. Absorbance was read on Luminometer (Flexstation III) at a wavelength of 450 nm. The Albumin amounts were calculated by standard curve and normalized by protein concentration (mg/ml).

3-[4,5-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay

To determine the non-cytotoxic concentrations of the test chemicals, the undifferentiated AT-MSCs were seeded at densities of 2 x 10^4 cells in 96-well plate and cell viability was measured by an

MTT assay (ATCC) after exposure of 16 h. After the inhibitory concentration 12.5 ($IC_{12.5}$) for each chemical was determined by the assay, serialdilutions (Ars; 0 to 2.5mM, AAP; 0 to 5mM) of the chemicals using the $IC_{12.5}$ as the highest dose were treated to cells. To evaluate toxicity during the hepatic differentiation process, AT-MSCs were seeded at densities of 4 x 10^3cells per well in Collagentype I-coated 96-well plate and the cells were treated with non-cytotoxic concentration 0 to 2.5mM of Ars and 0 to 5mM of AAP for 10days (from day 4 to day 13). Cytotoxicity was evaluated by MTT assay. MTT reagent was added and incubated at 37°C for 2 h. All supernatant was removed and DMSO was added into well. Absorbance was read at 570 nm and the results were expressed as a percent of non-treated control for each concentration.

Enzyme activities for cellular damage

To measure enzyme leaking by cellular damage, lactate dehydrogenase (LDH), Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activities were estimated using each enzymatic assay kit (Bioo Scientific Corp, USA) according to manufacturer's instruction. Briefly, at the final day of chemical treatment, the supernatant of each well (10 μl for ALT and AST analysis, and 5 μl for LDH) was transferred to 96-well plate and then Reagent mix (240 μl of ALT and AST, and 250 μl of LDH, respectively) was added. Absorbance was read at 340 nm and the results were expressed as Unit per Liter (U/L) by normalization of protein concentration (mg/ml).

Statistical analysis

Data were expressed as means ± SD for each group. The statistical significance of differences was determined using Statistica software. Statistical analysis was performed using Duncan test. Results were considered statistically significant at $*p<0.05$.

Results

Differentiation of adipose stem cells into hepatic lineage cells

To differentiate AT-MSCs to HLCs, stromal vascular fractions were isolated from human adipose tissues, and the AT-MSCs were purified by MACS using a CD105 antibody (Table 1). The purified cells showed a typical MSC morphology (Fig. 1a and b). Also, I verified that most of the sorted CD105-positive cells expressed CD13, CD29, CD44 and CD166 as MSC-specific antibodies by flow cytometer and the expressions of CD markers were not significantly different between abdomen and breast origin (data not shown). In this study, breast-derived AT-MSCs (53-year-old female) at passage 3 to 4 were mainly used for hepatic differentiation. During the hepatic differentiation process for 13 days, AT-MSCs showing a long slender shape changed into roundish angular polygonal-type cells (Fig. 2a). HLCs induced from AT-MSCs expressed hepatocyte-specific proteins such as AFP, ALB and CK18 at Day 13 (Fig. 2b). Similarly, the expressions of hepatocyte-specific genes, such as CCAAT-enhancer binding proteins alpha (C/EBPA), hepatocyte nuclear factor 4 alpha (HNF4A), GATA binding protein 4 (GATA4), AFP, ALB, CYP3A4 and CYP3A7, significantly ($p<0.05$) increased in HLCs, while the expression of CYP1A2 increased compared to AT-MSCs, but this difference was not significant (Fig. 2c). The glycogen storage gradually increased during the differentiation, and this effect was strong in HLCs at day 13 of differentiation (Fig. 3a). The activities of

CYP1A2 and CYP3A4 were measured in HLCs after 13 days of differentiation. As compared to AT-MSCs, the activity of CYP1A2 significantly increased by 4.3-fold (Fig. 3b) and that of CYP3A4 increased by approximately 2.5-fold in HLC (Fig. 3c). Also, albumin amount of HLCs was significantly increased by 14.2-fold as compared to that of AT-MSCs (Fig. 3d).

Cytotoxicity of Ars and AAP

To determine the cytotoxicity of test chemicals during the differentiation process, non-cytotoxic concentrations ($IC_{12.5}$) of Ars and AAP were administered to differentiating cells from day 4 to day 13. A MTT assay on day 13 showed that cell viability was decreased when either Ars or AAP was added to the cell culture system at concentrations exceeding 1 mM or 5 mM, respectively (Fig. 4a and b). To determine the cell damage, the levels of LDH, ALT and AST activity were estimated in the media after 13 days of treatment with Ars or AAP. The enzymatic activities in the Ars-treated group did not significantly differ by concentration (Fig. 5a). In the AAP-treated group (Fig. 5b), the LDH and AST activities increased in a dose-dependent manner, and this increase reached a statistical significance level ($p<0.05$) at 2.5 and 5 mM AAP. The ALT activity was significantly augmented ($p<0.05$) at 1.25, 2.5 and 5 mM of AAP examined.

Effect of chemicals on the expression of hepatic specific genes and CYP activity

The expression of ALB significantly decreased ($p<0.05$) in response to 0.5, 1 and 2.5 mM Ars (Fig. 6a). However, AFP expressions significantly changed their expression levels at 2.5 mM of Ars (Fig. 6b). In the AAP-treated group, the expressions of ALB and AFP significantly decreased ($p<0.05$) in a dose-dependent manner (Fig. 6c and d).

The activities of CYP1A2 and CYP3A4 were evaluated on day 13 in the groups treated with Ars and AAP. In the Ars-treated group, the CYP1A2 activity decreased significantly ($p<0.05$) for all concentrations (Fig. 7a), whereas that of CYP3A4 remained constant (Fig. 7b). In the AAP-treated group, the activity of CYP1A2 increased in a dose-dependent manner (Fig. 7c), but that of CYP3A4 remained unchanged in all treatment groups (Fig. 7d).

Table 1. Information of adipose-derived tissues collected from patients and MACS sorting by CD105 antibody.

Donor #	Tissue origin	Genotype/ Age (years)	# of cells loaded on MACS	After MACS separation	
				# of MACS+cells	Recovery ratio (%)
Donor # 1	Abdomen	XY/27	2.53 x 10^6	2.0 x 10^6	79.1
Donor # 2	Abdomen	XY/27	5.74 x 10^6	3.28 x 10^6	57.1
Donor # 3	Abdomen	XY/31	5.70 x 10^6	4.17 x 10^6	73.2
Mean ± SD					69.8 ± 11.3
Donor # 4	Breast	XX/45	2.56 x 10^6	0.2 x 10^6	7.8
Donor # 5	Breast	XX/53	7.25 x 10^6	1.28 x 10^6	17.7
Mean ± SD					12.7 ± 6.96

P = 0.0378 (t-test)

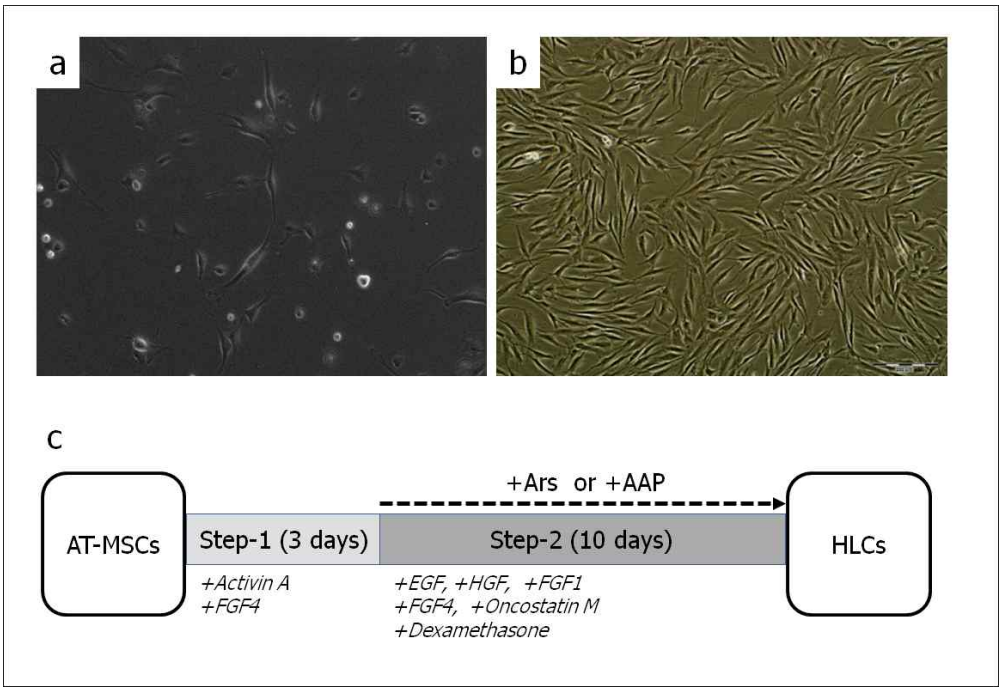

Fig. 1. Hepatic differentiation protocol with AT-MSCs. AT-MSCs were purified by MACS using the CD105 antibody. Undifferentiated AT-MSCs show a typical MSC shape and spindle morphology; 3 days after MACS separation (a) and passage 5 (b). The undifferentiated MSCs were induced to endodermal cells for 3 days (Step-1) and differentiated into HLCs via the addition of exogenous factors (Step-2) (c). To evaluate the chemical-induced toxicity, the cells were treated with Ars and AAP during Step-2 of differentiation.

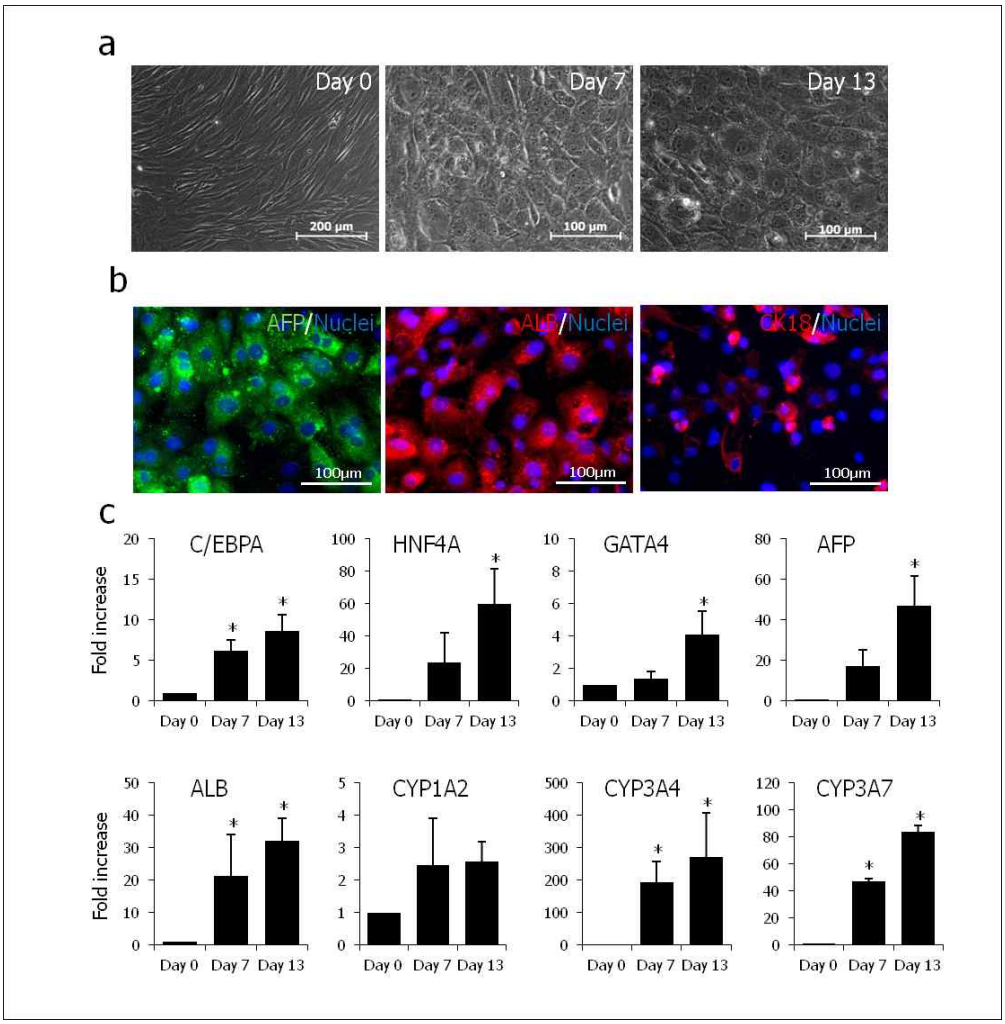

Fig. 2. Characterisation of the differentiation process from AT-MSCs to HLCs. AT-MSCs (Day 0) morphologically changed into HLCs (Day 7 and Day 13) (a). Hepatocyte-specific proteins of AFP, ALB and CK18 were expressed at Day 13 (b). Also, all of hepatic genes such as C/EBPA, HNF4A, GATA4, AFP, ALB, CYP1A2, CYP3A4 and CYP3A7 increased during the hepatic differentiation process (c). Values are the mean ± SD of three replications. *; significantly different from control at $p<0.05$.

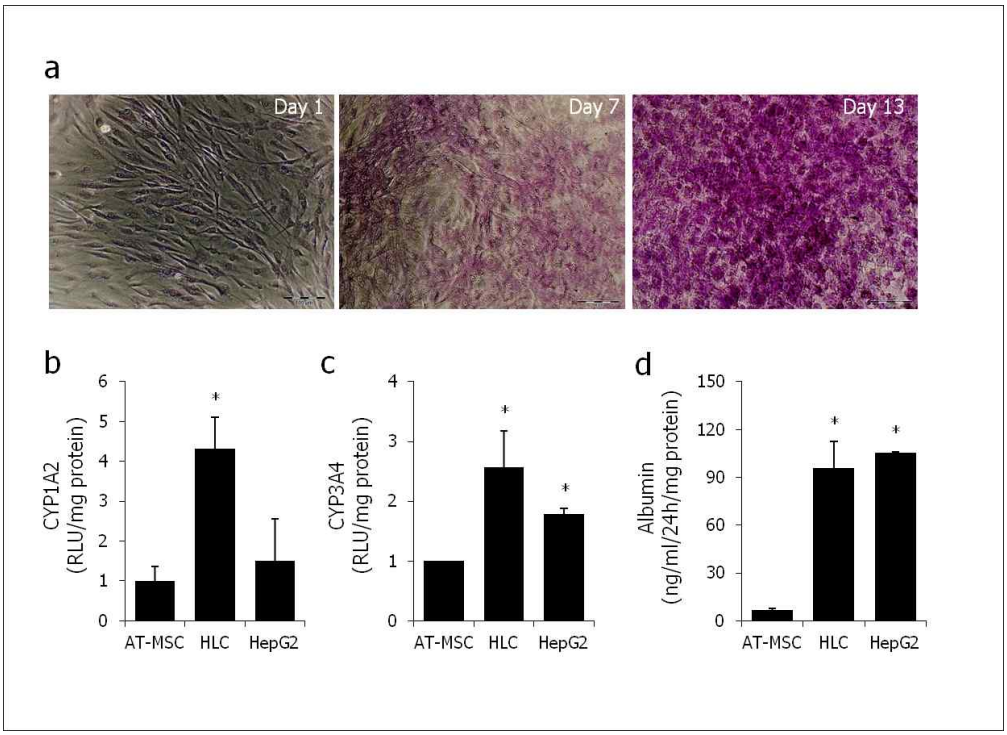

Fig. 3. Hepatic functionalities on AT-MSC derived hepatic differentiation. Glycogen storage was confirmed by PAS staining at Day 1, 7 and 13 (a). The activities of CYP1A2 (b) and CYP3A4 (c) significantly increased in HLCs compared to AT-MSCs. Also, albumins were significantly secreted in HLCs compared to AT-MSCs. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

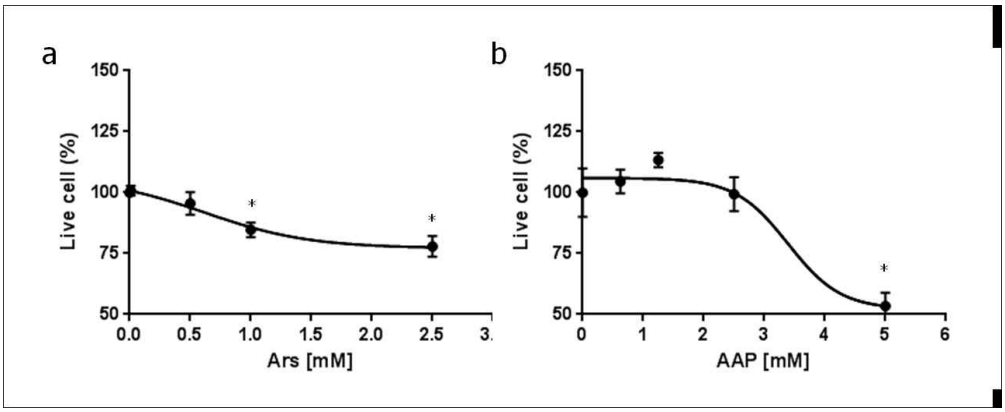

Fig. 4. MTT assay for non-cytotoxic concentrations of chemicals. After treatment of non-cytotoxic concentrations for 10 days of hepatic differentiation, 1 and 2.5 mM Ars (a) and 5 mM AAP (b) were cytotoxic to HLCs compared to the non-treated control group. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

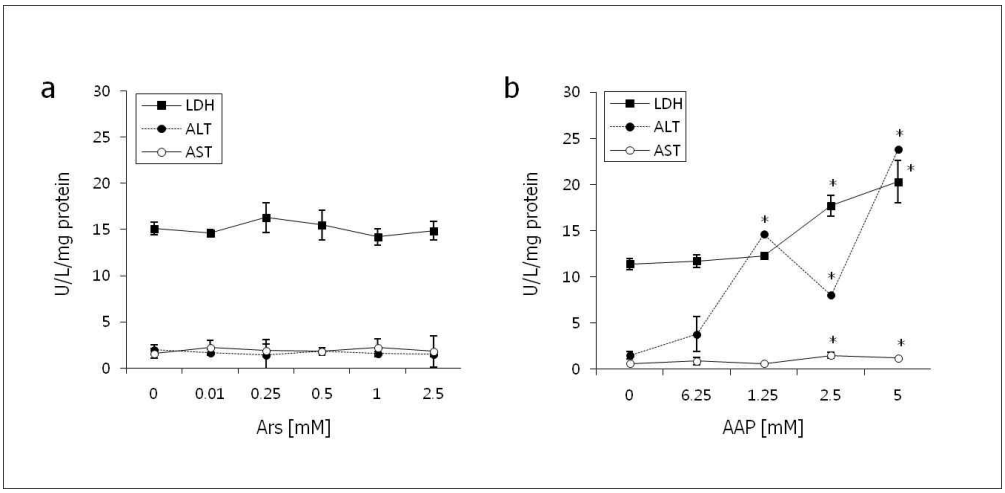

Fig. 5. Enzymatic effects after the treatment with Ars and AAP. Cellular damage was estimated by LDH, ALT and AST after treatment with Ars (a) and AAP (b) for 10 days of hepatic differentiation. Enzyme activities were expressed as Unit per Liter (U/L) and the values were normalized by protein concentration (mg/ml). Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

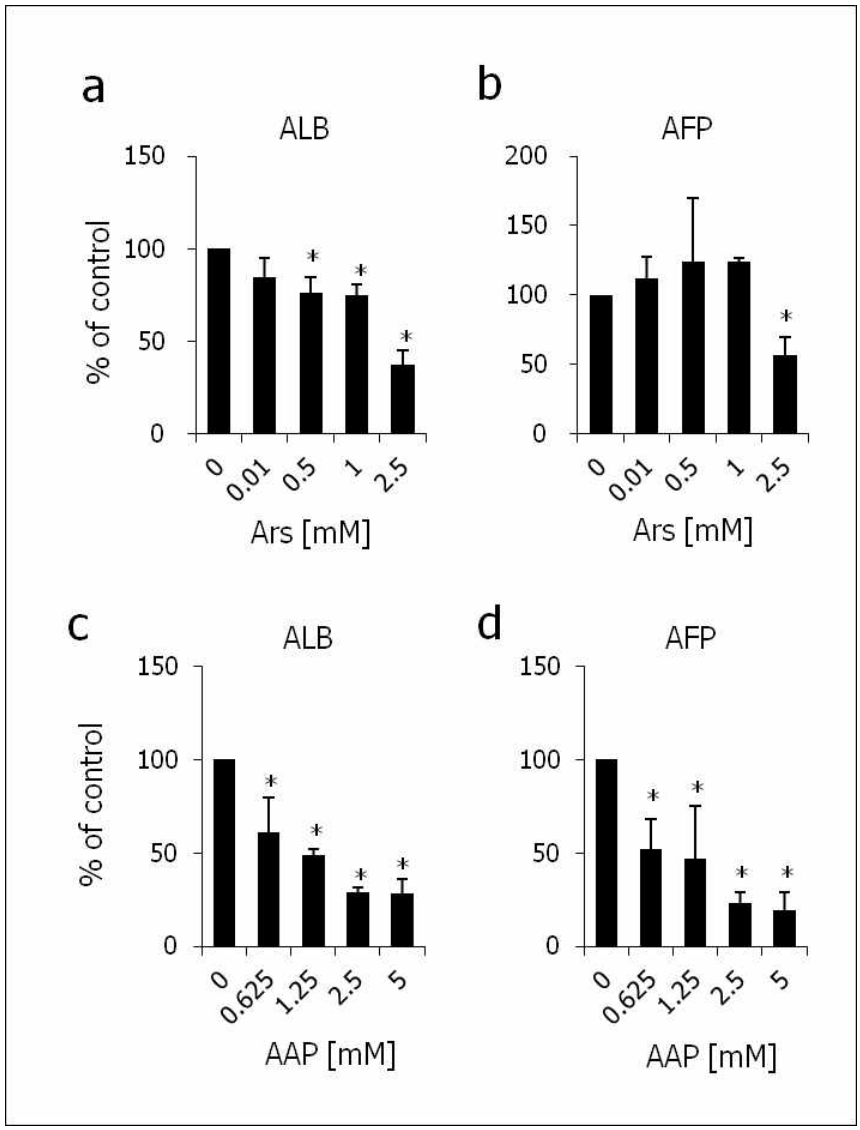

Fig. 6. Expressions of hepatocyte-specific genes after treatment with Ars and AAP. The expressions of ALB and AFP were evaluated in response to Ars (a and b) and AAP treatment (c and d). Data are expressed as the fold increase in the mRNA level compared to the non-treated group. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

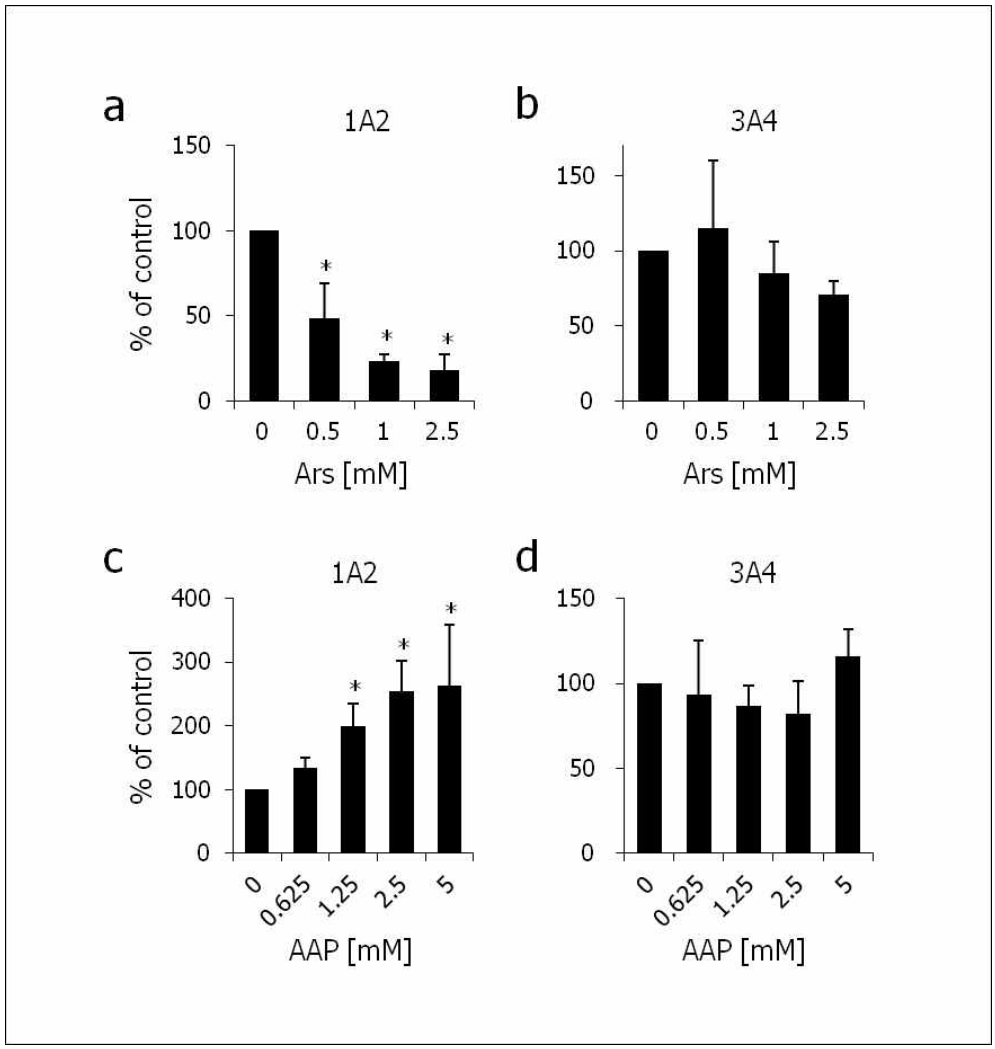

Fig. 7. CYP activities after treatment with Ars and AAP. CYP1A2 significantly decreased in a dose-dependent manner in response to Ars (a) while it significantly increased in response to AAP (c). The CYP3A4 activities did not significantly differ between both chemicals (b and d). Data are expressed as the fold increase compared to the non-treated group. Values are mean ± SD of three replications. *; significantly different from control at $p<0.05$.

Discussion

HLCs derived from human stem cells (hESC, hiPSC and adult stem cell) have been applied to evaluate hepatotoxicity, which is a leading cause of drug failure at the developmental stages (Mandenius et al, 2011;Rodrigues et al, 2014;Sirenko et al, 2014). Recently, AT-MSCs have been suggested as a material of hepatic induction (Aurich et al, 2009;Banas et al 2007;Okura et al, 2010b). AT-MSC derived HLCs have potential advantages as candidates for hepatotoxicity tests of chemicals, some of which are the proliferation ability and the capability to differentiate into other cell types. Particularly, they are a readily available source of human cells. Therefore, the AT-MSCs were applied in the hepatic differentiation and investigated the effect of Ars and AAP on the hepatic differentiation process in the present study.

In this study, AT-MSCs were differentiated to HLCs for 13 days. Hepatic genes and proteins, glycogen synthesis and CYP enzyme activity of HLCs were gradually increased during the differentiation. In the preliminary study, the instability of hepatic markers and a large cell death at the extended culture (day 17) were founded. Therefore, the hepatic differentiation duration was shortened to overcome such drawbacks. Nonetheless, the hepatic functionalities might be not completely enriched in HLCs at day 13, it can be an available model to evaluate the developmental toxicity of compounds because Step-2 sufficiently showed the developing stage of endodermal cells to HLCs.

The metabolic enzymes ALT and AST are released into the blood in response to liver damage, and elevated levels of these enzymes are associated with liver injury. LDH is a ubiquitously expressed intracellular enzyme that is associated with organ toxicity. Significantly elevated levels of these enzymes (normal ranges; 0-45 IU/L of ALT and 0-35 IU/L of AST) suggest a medical problem, such as liver damage (Limdi and Hyde 2003). Therefore, these enzymes can be used as indicators to evaluate the cytotoxicity of drugs and chemicals. In this study, 5 mM AAP was cytotoxic to HLCs according to a MTT assay and liver enzymes (LDH, ALT and AST) were elevated in a dose-dependent manner in the AAP-treated group. These findings demonstrate that increasing the AAP concentration damaged the cells. Ars was cytotoxic to HLCs at concentrations exceeding 1 mM but did not significantly change the enzyme activities. In a previous study, Kang et al. suggested that hepatic progenitor cells (HPCs) were more susceptible to Ars-derived damage than HLCs, and Ars might be detoxified by the CYP system during the HLC differentiation process (Kang et al, 2013). However, the evaluation was not conducted whether the chemical-induced cytotoxicity was derived from metabolites generated by CYP enzymes or not. The CYP enzymes might be not completely expressed during the induction period. The differentiating HLCs, still lack of CYP expression, could be directly killed by AAP and Ars. According to a report, AAP could induce cell death by necrotic pathway (McGill et al, 2012). The leaking of LDH, ALT and AST was not sufficient to distinguish between apoptotic and

necrotic cell death. Therefore, it will need to investigate necrotic cell death induced by AAP and Ars in the hepatic development.

AFP is expressed very early during embryonic liver development, and ALB is elevated during the hepatic maturation of embryonic development. In the present study, Ars (0.5, 1 and 2.5 mM) inhibited the expression of ALB but only 2.5 mM of Ars affected on the expression of AFP, whereas AAP inhibited the expressions of ALB and AFP at all non-cytotoxic concentrations. This experiment suggests that Ars and AAP could affect the albumin synthesis/secretion during the differentiation process. Furthermore, AAP may affect the early hepatic cell lineage during differentiation, even at non-cytotoxic concentrations. The present study also suggests that AFP and ALB could act as markers in an in vitro hepatotoxicity test that uses AT-MSC derived HLCs.

The development of non-tumorigenic cell lines that show a hepatocyte phenotype would allow for the substitution of primary hepatocytes as the cell model of CYP induction, xenobiotic biotransformation and hepatotoxicity. Furthermore, the high inducibility of CYP transcripts could serve as a sensitive model for profiling the xenobiotic-induced expression of CYP (Sa-ngiamsuntorn et al, 2011). This present experiments demonstrated that AT-MSC derived HLCs contained CYP enzymes, such as CYP1A2 and CYP3A4. Even though the enzymes may be not completely in AT-MSC derived HLCs at day 13 of differentiation, both activities were higher than HepG2 cells. Therefore, both CYP enzymes were used as marker for detecting

adverse effects of Ars and AAP on the hepatic development.

Taken together literature reviews, there is still some controversy on the roles of CYP1A2 and CYP3A4 for the hepatotoxic effect of AAP. The hepatotoxicity of AAP is due to the production of a toxic metabolite, A-acetyl-P-benzoquinone imine (NAPQI), produced by CYP450 enzymes (Dahlin et al, 1984). A study using a human recombinant CYP enzyme reported that CYP3A4 had the highest bioactivation capacity at both the therapeutic 50 μM and toxic 1 mM concentrations (Laine et al, 2009). However, human volunteer studies using a therapeutic dose of AAP did not show enhanced NAPQI formation following CYP3A4 induction (Manyike et al, 2000). A study of human liver microsomes showed that CYP1A2 played a major role in AAP bioactivation and toxicity at higher concentrations of AAP (Patten et al, 1993). Another human study suggested that CYP1A2 inducers do not increase the production of NAPQI after a therapeutic dose of AAP (Sarich et al, 1997). In an animal model, CYP1A2 was involved in AAP metabolism but contributed significantly to the bioactivation and toxicity when AAP was given at high doses (Snawder et al, 1994a). Others reported that CYP1A2 does not play a significant role in AAP hepatotoxicity in mice (Tonge et al, 1998). CYP1A2 contributes more to the bioactivation and toxicity of AAP at high doses (Snawder et al, 1994b). In this study, AAP treatment did not change the CYP3A4 activity while the CYP1A2 activity was elevated in a dose-dependent manner in the AAP-treated group. The present finding also shows that CYP3A4 is not likely to contribute

AAP metabolism in the presently examined cells and the production of NAPQI may be increased via the CYP1A2 enzyme, which may result in the cellular damage of HLCs.

The metabolic fate and toxicity of Ars compounds vary according to the type of compound and methylation reaction in the liver. Organic arsenic compounds, such as Ars, are considered less toxic (1980;Hughes et al, 2011). I did not find many reports on the effect of Ars on the induction of hepatic enzymes. CYP1A2 is induced by polyaromatic hydrocarbon compounds that have a high affinity for the aryl hydrocarbon receptor and play an important role in metabolising the compound. Certain polychlorinated dibenzo-p-dioxins and coplanar polychlorinated biphenyls reportedly inhibit the function of CYP1A2 (Chen et al, 2003). In the present study, Ars did not affect CYP3A4 like AAP, but it decreased CYP1A2. This finding suggests that Ars could potentiate the toxicity of compounds that are metabolised by CYP1A2. Alternatively, Ars could decrease the toxicity of chemicals whose toxicity is mediated via the metabolic activation induced by CYP1A2.

Collectively, the above data and the present in vitro data using HLCs derived from AT-MSCs support that AAP could have a more toxic effect on the hepatic system via increasing CYP1A2, inhibiting AFP and increasing hepatic enzymes. The present study showed that the present protocol mimics embryonic development and may be applied to evaluate the effect of chemicals during hepatic development.

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국문 요약문

인체 줄기세포에서 유래된 간세포는 신약개발 단계에서 약물로 인한 간장독성의 평가를 위해 사용되어 왔으며, 지방조직 유래 중간엽 줄기세포에서 직접 유도된 간세포는 간장독성 평가를 위한 세포로서 많은 장점을 가지고 있다. 본 실험에서는 인체 지방조직 유래 중간엽 줄기세포를 분리하여 간세포로 분화유도하고 비소와 아세트아미노펜이 간세포분화에 미치는 영향을 평가하였다. 인체 지방조직 유래 중간엽 줄기세포의 간세포로의 분화를 간세포 특이단백질 발현, 간세포 특이 유전자 발현, 약물대사효소인 CYP1A2, CYP3A4 활성 증가, glycogen 축적, albumin 분비량 증가로 확인하였다. 간세포 분화과정 중에 비소와 아세트아미노펜이 미치는 영향을 평가하기 위해, 간세포로 분화중인 세포에 약물(IC12.5이하)을 분화 4일째부터 13일까지 처리하였다. 약물이 첨가된 배지로 10일 동안 분화 유도 후 실험이 종료된 시점에서 alanine aminotransferase, lactate dehydrogenase, aspartate aminotransferase를 측정한 결과 비소 처리군에서는 실험농도 간 유의성 있는 차이가 나타나지 않았고, 아세트아미노펜 처리군은 농도 의존적으로 현저히 증가하는 경향이 있었으며 2.5mM과 5mM에서는 유의한 증가를 나타냈다. 약물로 인한 간세포 특이 유전자 발현을 확인한 결과 비소 처리군 0.5mM이상에서 albumin 발현이 유의성 있게 감소하였고 α-feto protein은 2.5mM에서 유의성 있는 감소를 나타냈다. 아세트아미노펜 처리군에서 간세포 특이 유전자 발현을 확인한 결과 albumin과 α-feto protein이 0.625mM이상의 모든 실험 농도에서 농도 의존적으로 유의한 감소를 나타냈다. CYP3A4 활성도는 비소와 아세트아미노펜에 의해 영향을 받지 않았다. CYP1A2 활성도는 아세트아미노펜 처리 군에서 증가한 반면, 비소 처리 군에서는 감소하였다. 결론적으로 지방

줄기세포 유래된 간세포는 약물의 간장독성을 평가하기 위한 효율적인 수단으로 사용될 수 있으며 아세트아미노펜은 비소와 비교했을 때 간세포 분화과정에 독성을 일으킬 수 있다고 사료된다.

핵심어: 인체 지방조직, 중간엽 줄기세포, 간세포, 간장독성, 비소, 아세트아미노펜

학번: 2010-21637