



## Placenta extract promote liver regeneration in CCl<sub>4</sub>-injured liver rat model

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### ARTICLE INFO

#### Article history:

Received 3 November 2010

Received in revised form 6 January 2011

Accepted 9 February 2011

Available online 24 February 2011

#### Keywords:

Placenta extract

Liver injury

Liver regeneration

Immunomodulation

### ABSTRACT

The human placenta is an organ for fetus development and abundant reservoir of various bioactive molecules. Interest to human placenta extract (hPE) is growing, and application with trial of hPE is widening in oriental medicine including in liver diseases. However, underlying mechanisms for therapeutic effects are still unclear. Here, we investigated therapeutic effects of hPE in carbon tetrachloride (CCl<sub>4</sub>)-injured rat liver model in vivo and in damaged rat hepatic cells exposed to CCl<sub>4</sub> in vitro. In addition, regulation of inflammatory responses by treatment of hPE was investigated. Serum levels of GOT/AST and GPT/ALT were significantly reduced ( $P < 0.05$ ), and uptake/excretion of indocyanine green in serum was significantly induced at 3 weeks after intravenous hPE administration in CCl<sub>4</sub>-injured rat model ( $P < 0.05$ ). Expression of type I collagen (Col I) and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) was decreased, whereas that of matrix metalloproteinase-9 (MMP-9) was increased resulting in improvement of score for fibrotic grade in hPE group. Also, albumin, proliferation activities and molecules associated with liver regeneration (e.g. interleukin-6, gp130, ATP binding cassette transporters, cyclin A) were more increased in hPE administration group than Non-hPE group. hPE administration suppressed activated T-cell proliferation via increasing anti-inflammatory cytokines and decreasing pro-inflammatory cytokines. These results suggest that hPE could be effective for liver disease through reduction of fibrosis, induction of liver regeneration, and regulation of inflammatory responses. These findings are important for understanding the roles of hPE and provide evidences for therapeutic effects of hPE in hepatic diseases which could lead to potential clinical applications.

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### 1. Introduction

Liver fibrosis represents wound healing process responsive to chronic liver injury. Advance in liver fibrosis leads to cirrhosis and eventually liver failure which greatly accounts for morbidity and mortality [1,2]. Although orthotopic liver transplantation is currently the only definitive therapeutic option for end-stage liver disease, it has limitations including lack of donor organs, invasiveness of procedure, poor long-term graft survival, requirement of lifelong immunosuppression, and high costs [3,4]. Thus, search for alternative effective therapies and validation of their feasibility are of need.

**Abbreviations:** CCl<sub>4</sub>, carbon tetrachloride; Col I, type I collagen; GOT/AST, glutamate-oxaloacetate aminotransferase; GPT/ALT, glutamate-pyruvate aminotransferase; hPE, human placenta extract; i.p., intraperitoneally; ICG, indocyanine green; Ki-LI, Ki-67 labeling index; N-hPE, naïve hPE; P-hPE, pasteurized hPE.

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The placenta is usually discarded after delivery. During pregnancy, however, it is an important link between mother and fetus providing various nutrients. In addition, the placenta is a rich source of many biological components including hormones, cytokines, chemokines, and growth factors such as hepatocyte growth factor, epidermal growth factor, and transforming growth factor- $\alpha$ , - $\beta$  of which receptors have been identified in human placenta [5–7]. Many of these factors possibly act in an autocrine and/or paracrine fashion within the human placenta and regulate production of other biologically active substances, which may hold potentials for therapeutic agents as suggested in previous studies where fractions of human placenta extract (hPE) stimulated tissue repair processes [5,8,9]. hPE has been traditionally evaluated and used to enhance wound healing, replenish vital essence, and aid in treatment of psoriasis, rheumatoid arthritis, and others in Oriental medicine [5,10,11]. Nowadays, aqueous hPE is available and licensed for post-surgical dressings, burn injuries, and chronic wounds in many countries and for liver disease in some countries [5,12–14]. Although the usage of hPE is widening, limited information is available about the mechanisms regarding its therapeutic effects, except for some reports that hydrolyte or extract of placenta stimulated liver regeneration in rats [15].

Effective components present in placental extract can vary according to the method of preparation [5], because the efficacy of placenta extract is highly dependent on manufacturing steps which include excessive digestion, organic phase extraction, and autoclave/pasteurization processes. Since these manufacturing steps can denature effective and functional molecules derived from placenta, the efficacy of placental extract can be limited. To overcome these potential problems, we previously showed and suggested a better way of placental extraction to preserve several bioactive molecules by means of a water-soluble method [16].

Based on these backgrounds, we investigated the effects of hPE on liver disease model using CCl<sub>4</sub>-injured rat *in vivo* and damaged rat hepatic cells *in vitro*. We also investigated the effects of hPE on regulation of inflammatory cytokines in activated T-cells. hPE was extracted by two different water-soluble methods to produce naïve hPE (N-hPE) and pasteurized hPE (P-hPE), as described below, and the effects were compared between them.

## 2. Materials and methods

### 2.1. Preparation of hPE

Full term placentas were collected after obtaining written informed consent from the donors. The collection of samples and their utilization for research purposes were approved by the Institutional Review Board of CHA General Hospital, Seoul, Korea. hPE was prepared with water-soluble methods as follows. Red blood cells were removed from placental tissue by washing buffer (50 mM Tris-HCl; 150 mM NaCl; 150 mM Sucrose, pH 7.2) for 30 min each at 4 °C. The washed tissue was chopped using sterile scissors. The tissue was snap-frozen in liquid nitrogen for additional grinding, thawed and resuspended in 30 ml of homogenizing buffer (DMEM high glucose; 0.32 M sucrose; 2 mM EDTA), which was then sonicated on ice (amplitude 60, pulse 4, and repeat 4). After centrifugation (4500 rpm for 15 min at 4 °C), total protein content of the hPE was measured using a BCA™ protein assay kit (Pierce Chemical, Rockford, IL, USA). N-hPE was filtered without heating and P-hPE was filtered after heating for 30 min at 56 °C by 0.22-µm filter. hPE was used fresh or frozen in liquid nitrogen and stored at –80 °C until usage. The components in N-hPE and P-hPE including cytokines, chemokines and growth factors were analyzed by multiplex supernatant cytokine assay [16]. Many cytokines, chemokines, and growth factors, known as essential factors for cell to cell interactions, were detected in hPE demonstrating that hPE contained abundant molecules of biological activity, and specifically, N-hPE contained higher levels of several cytokines and growth factors compared to P-hPE.

### 2.2. Animals and materials

Six-week-old male Sprague–Dawley rats were used. Rats were maintained in an air-conditioned animal house with specific pathogen-free conditions and allowed unlimited access to chow and water. For construction of CCl<sub>4</sub>-injured rat model, CCl<sub>4</sub> (1.6 g/kg; Sigma, St. Louis, MO, USA) dissolved in corn oil was injected intraperitoneally (i.p.) twice a week for 9 weeks (n = 27). CCl<sub>4</sub> 9 weeks group (n = 6; CCl<sub>4</sub> group) and control group without treatment of CCl<sub>4</sub> (n = 5; control group) were sacrificed and other CCl<sub>4</sub> 9 weeks treated rats were randomly divided into 3 groups; either 1 mg/ml N-hPE (n = 7; N-hPE group), 1 mg/ml P-hPE (n = 7; P-hPE group), or none (n = 7; Non-hPE group) was injected through the tail vein once a weeks for 3 weeks. At the end of experiment, blood and liver tissue of rats were collected both in hPE and Non-hPE groups for blood chemistry analysis, reverse transcription polymerase chain reaction (RT-PCR) analysis, Western blot analysis, gelatin zymography analysis, and histopathological analysis. We conducted all animal experimental procedures using protocols approved by the National Institutes of Health Guidelines.

### 2.3. Blood chemistry analysis

Blood was collected from all groups of animals at 3 weeks after hPE injection. Glutamate-oxaloacetate aminotransferase (GOT/AST) and glutamate-pyruvate aminotransferase (GPT/ALT) were measured using an auto biochemistry detector (DRY-CHEM 3500i, Fujifilm, Korea). In order to analyze liver function, indocyanine green (ICG) uptake and excretion assay were performed. ICG was dissolved in sterile water (0.5 mg/ml) and administered intravenously [17]. Blood was obtained from the ventricular artery after 15 min, and the absorbance was measured at 805 nm using a spectrophotometer.

### 2.4. MTT analysis

WB-F344 (rat hepatic epithelial stem-like cells) or T-HSC/Cl-6 (rat hepatic stellate cells transformed by simian virus 40) were cultured  $5 \times 10^3$  cells per well on 96-well plate (BD Falcon, BD Bioscience Discovery Labware, Bedford, MA, USA) for 24 h. The cells were then treated with 0, 3, 6, 9, 12, and 15 mM CCl<sub>4</sub> dissolved in dimethyl sulfoxide (DMSO) and incubated for 3 h, which is followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) analysis. Culture media were replaced with MTT (Sigma) contained media (0.5 mg/ml) and incubated for 3 h. The absorbance was measured at 595 nm after being dissolved in 200 µl of DMSO. Experiments were performed in triplicate.

### 2.5. *In vitro* proliferation analysis

WB-F344 or T-HSC/Cl-6 were cultured  $1.5 \times 10^4$  cells per well on 24-well culture plate (BD Biosciences) for 24 h, and then the cells were treated with 6 mM CCl<sub>4</sub> for 3 h at 37 °C. After CCl<sub>4</sub> treatment on WB-F344 or T-HSC/Cl-6, the cells were washed with media for 3 times and cultured with media containing N-hPE or P-hPE (0, 40, 80 and 160 µg/ml of media) for 24 h. Proliferation of WB-F344 or T-HSC/Cl-6 was determined with BrdU ELISA kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. No treatments of CCl<sub>4</sub> and hPE were used in controls, and this experiment was performed in triplicate.

### 2.6. T-cell proliferation analysis

To assess the ability of N-hPE to suppress T-cell proliferation, human umbilical cord blood (UCB) mononuclear cells were prepared by centrifugation on a Ficoll Hypaque density gradient, and  $2 \times 10^5$  cells were cultured per well in 96-well culture plate in the absence or presence of 40 µg/ml hPE with or without 1 µg/ml anti-CD3 and anti-CD28 T-cell activating mAbs (eBioscience, Inc., San Diego, CA, USA) for 48 h and 72 h. To analyze the proliferative response of T-cell clonal expansion, BrdU ELISA (Roche) was performed according to the manufacturer's protocol at 48 h cultivation, and clustering of T-cells was confirmed in bright field at 72 h cultivation.

### 2.7. Multiplex supernatant cytokine assay

The supernatant of T-cells was harvested in the absence or presence of 40 µg/ml hPE with or without 1 µg/ml anti-CD3 and anti-CD28 activating mAbs after 72 h cultivation. For measurement of Flt3L, fractalkine, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-12 (p40), sCD40L, TNF-α, and 50 µl of supernatant were combined with coated beads in MILLIPLEX™ MAP kit (Millipore Corp., Billerica, MA., USA). Commercial kits were run in individual plates with buffers and standards according to the previous reports [18]. Incubations and washes were done in 1.2-µm filter membrane 96-well microtiter plates (Millipore Corp.). After the final wash, beads from the 96-well microtiter plates were resuspended in a 125 µl cuvette of a Luminex instrument. An acquisition gate was set between 7500 and 13,500 for a

doublet discriminator, sample volume was 75  $\mu$ l, and 100 events/region were acquired. Raw data (mean fluorescence intensity) from all the bead combinations tested were analyzed with Master Plex QT3.0 quantification software (MiraiBio Inc., Alameda, CA, USA) in order to obtain concentration values. IL-10 and IL-13 were measured using multiplex detection kits (Bio-Rad Laboratories, Richmond, CA, USA). The assay was performed according to the protocol, except that all samples were centrifuged for 20 s at 14,300 rpm to remove debris, and 50  $\mu$ l of each sample was diluted at a ratio of 1:3 in sample diluent. All samples were assayed in duplicate and analyzed with a Luminex 200 Labmap system (Luminex, Austin, TX, USA). Data analyses were performed using Bio-Plex Manager software version 4.1.1 (Bio-Rad Laboratories). Cytokine/chemokine concentrations were interpolated from an appropriate standard curve.

## 2.8. RT-PCR analysis

For RT-PCR analysis, rat liver tissues were homogenized and lysed in 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA). A reverse transcription reaction was performed with 1  $\mu$ g of total RNA and SuperScript™ III reverse transcriptase (Invitrogen). The cDNA was amplified using h-Taq DNA polymerase (Solgent, Korea). The primer sequences were: type I collagen (Col I) forward primer; 5'-CATCTCAGAAGCAGAATCTCC-3', Col I reverse primer; 5'-CCATAAACCACACTATCACCTC-3', 28s rRNA forward primer; 5'-TTGAAAATCCGGGGGAGAG-3', 28s rRNA reverse primer; 5'-ACATTGTCCAACATGCCAG-3'. Amplification reactions were performed on the following conditions: denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50–60 °C for 40 s, and elongation at 72 °C for 5 min. The PCR products were visualized and photographed following electrophoresis on 1% (w/v) agarose gel containing 0.5  $\mu$ g/ml ethidium bromide.

## 2.9. Western blot analysis

Rat livers from each group were homogenized and lysed with protein lysis buffer (Intron Biotechnology, Korea). Equal concentration of protein from individual rat was pooled in each group. A total of 50  $\mu$ g of protein lysate was loaded onto a 4–15% native gel (Bio-Rad Laboratories) for detection of Col I and onto a 10 or 12% sodium dodecyl sulfate (SDS) polyacrylamide gel for the detection of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), matrix metalloproteinase-9 (MMP-9), albumin, interleukin-6 (IL-6), gp130, cyclin A, ATP binding cassette transporter G1 (ABCG1), ABCG2, and actin. After electrophoresis, the protein was transferred onto a PVDF membrane (Millipore Corp.) at 100 V for 90 min by electro-elution. The membrane was incubated with an anti-Col I monoclonal antibody (1:5000; Abcam, Cambridge, UK), anti- $\alpha$ -SMA monoclonal antibody (1:5000; Abcam), anti-MMP-9 polyclonal antibody (1:1500; R&D systems, Minneapolis, MN, USA), anti-albumin monoclonal antibody (1:5000; Sigma-Aldrich), anti-IL-6 polyclonal antibody (1:800; Abcam), anti-gp130 polyclonal antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-cyclin A polyclonal antibody (1:1000; Abcam), anti-ABCG1 polyclonal antibody (1:500; Novus Biologicals, LLC Littleton, CO, USA), anti-ABCG2 monoclonal antibody (1:1000; Millipore Corp.), and anti-actin monoclonal antibody (1:1000; Santa Cruz Biotechnology Inc.) overnight at 4 °C. The secondary antibody reaction was performed with horseradish peroxidase-conjugated anti-mouse IgG (1:5000; Bio-Rad Laboratories) or anti-rabbit IgG (1:5000; Bio-Rad Laboratories) for 1 h at room temperature (RT) in an orbital shaker. After adequate washes, the bands were detected by enhanced chemiluminescence reagent (Amersham Biosciences, Arlington Heights, IL, USA).

## 2.10. Gelatin zymography

MMPs activity was determined by gelatin zymography analysis. Each pooled tissue protein sample (5  $\mu$ g) was loaded onto a 12%

polyacrylamide gel containing 0.1% gelatin under non-reducing condition. After electrophoresis, gels were renatured with renaturation buffer (Bio-Rad Laboratories) for 30 min at RT and incubated with development buffer (Bio-Rad Laboratories) for 16 h in 37 °C. The gels were subsequently stained with 0.5% Coomassie brilliant blue G-250 dye solution for 1 h at RT and were destained with buffer consisting of 30% methanol, 10% acetic acid, and 60% deionized water for 3 h to visualize the zymogen bands. The zymography gels were scanned, and molecular size of bands displaying enzymatic activity was estimated by prestained standard protein molecular weight markers (GenDEPOT Inc., Houston, TX, USA).

## 2.11. Histopathological analysis

Collected liver samples were fixed in 10% (v/v) neutral buffered formaldehyde, embedded in paraffin, and dehydrated with a graded ethanol series. Serial 5- $\mu$ m sections of the right lobe of liver were stained with hematoxylin & eosin (H&E) stain and Masson Trichrome (MT) to analyze structures and detect collagen deposition in liver tissues, respectively. The fibrotic grade of liver was scored by histopathological criteria, modified 0 to 4 as follows [19]: 0 representing no fibrosis, 1 representing fibrosis confined to enlarged portal zones, 2 representing periportal or portal–portal septa with intact architecture, 3 representing architectural distortion (septal fibrosis, bridging) without obvious cirrhosis, and 4 representing probable or definite cirrhosis.

## 2.12. Immunohistochemical analysis

Liver tissues were fixed, embedded in paraffin, and serially sectioned with 5- $\mu$ m of the right lobes of the livers. Sections were stained with Ki-67 monoclonal antibody (1:50; Novocastra, Newcastle, UK) and counterstained with H&E. The Ki-67 labeling index (Ki-LI) represented the percentage of hepatocytes with Ki-67-positive nuclei relative to the total number of hepatocytes in randomly selected sections, 6 fields for each rats under  $\times$ 100 magnification.

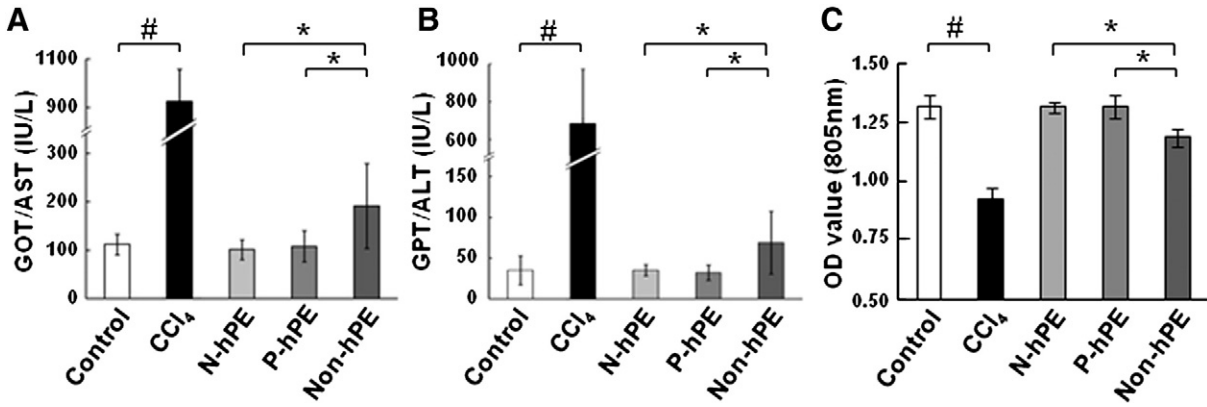
## 2.13. Statistical analysis

Results are presented as the means  $\pm$  SD. Statistical significance measured multiple comparisons were performed using the *t*-test with a significance level of  $P < 0.05$ .

## 3. Results

### 3.1. hPE improved liver function in CCl<sub>4</sub>-injured rat model

To investigate whether hPE administration had therapeutic effects in liver disease, we generated CCl<sub>4</sub>-injured rat liver which was compatible with early cirrhosis model confirmed by blood chemistry and pathological analysis [20]. The CCl<sub>4</sub>-injured rats were then randomly divided into 3 groups; N-hPE administered group ( $n = 7$ ), P-hPE administered group ( $n = 7$ ), and Non-hPE group for control ( $n = 7$ ). We analyzed GOT/AST and GPT/ALT levels in serum to evaluate the effects of hPE administration on the CCl<sub>4</sub>-injured rats at 3 weeks after hPE administration. As shown in Fig. 1A and B, the baseline levels of GOT/AST and GPT/ALT in CCl<sub>4</sub>-injured rats were significantly higher compared to the control group ( $P < 0.001$ ), and the levels decreased significantly more in both N-hPE and P-hPE groups than Non-hPE group ( $P < 0.05$ ). In addition, to investigate whether hPE administration could improve liver function, ICG uptake/excretion assay was performed (Fig. 1C). Uptake/excretion of ICG in CCl<sub>4</sub>-injured rats were significantly lower compared to the control group ( $P < 0.001$ ), and it increased significantly more in N-hPE and P-hPE groups than Non-hPE group ( $P < 0.05$ ). These data demonstrated that N-hPE and P-hPE could improve liver function of CCl<sub>4</sub>-injured rats.



**Fig. 1.** Blood analyses in each group. For construction of CCl<sub>4</sub>-injured rats, CCl<sub>4</sub> dissolved in corn oil was given twice a week for 9 weeks in the CCl<sub>4</sub> group (n = 6), and equal volume of corn oil alone was given in the control group (n = 5). After 9 weeks of CCl<sub>4</sub>, either 1 mg/ml naïve human placenta extract (n = 7; N-hPE group), 1 mg/ml pasteurized hPE (n = 7; P-hPE group), or none (n = 7; Non-hPE group) was injected. (A,B) The serum levels of GOT/AST and GPT/ALT in hPE groups decreased significantly compared to Non-hPE group. <sup>#</sup>P<0.05 statistically significant compared to CCl<sub>4</sub> group; <sup>\*</sup>P<0.05 statistically significant compared to Non-hPE group. (C) Uptake/excretion of ICG measured at 805 nm significantly increased in hPE groups compared to Non-hPE group. <sup>#</sup>P<0.001 statistically significant compared to CCl<sub>4</sub> group; <sup>\*</sup>P<0.05 statistically significant compared to Non-hPE group. Data were expressed as means ± SD.

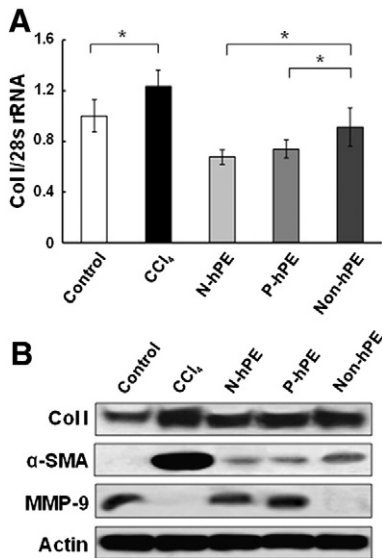
**3.2. hPE reduced Col I and α-SMA expression in CCl<sub>4</sub>-injured rat liver**

In order to confirm the effects of hPE on Col I and α-SMA expression in CCl<sub>4</sub>-injured rats, we performed RT-PCR and Western blot analysis. The mRNA expression of Col I in CCl<sub>4</sub> group was significantly increased compared to the control group (P<0.001) and decreased significantly more in both N-hPE and P-hPE groups than Non-hPE group (P<0.05; Fig. 2A). In addition, we analyzed the pattern of protein expression of Col I, α-SMA, and MMP-9, well known as a regulator of degradation of extra cellular matrix (ECM) components including collagen [21,22], using Western blot analysis. The expression of Col I and α-SMA was decreased in both N-hPE and P-hPE administrated groups compared to the CCl<sub>4</sub>

group and Non-hPE group. In contrast to the disappearance of expression of MMP-9 in CCl<sub>4</sub> group and Non-hPE group, MMP-9 was dramatically increased in both N-hPE and P-hPE groups (Fig. 2B). These data demonstrated that N-hPE and P-hPE administration could decrease Col I and α-SMA expression in CCl<sub>4</sub>-injured rat liver.

**3.3. hPE reduced collagen deposition in CCl<sub>4</sub>-injured rat liver**

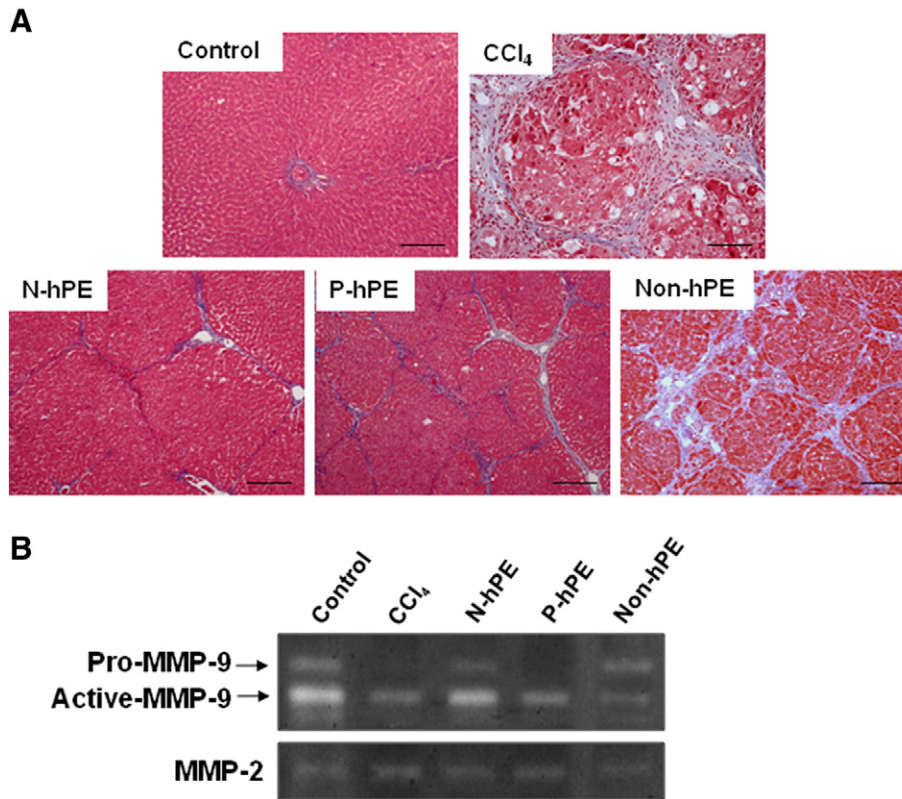
To investigate whether hPE could decrease the abnormal hepatic accumulation of ECM, MT staining was performed (Fig. 3A). The collagen deposition and fatty change were excessively increased in CCl<sub>4</sub> treatment group for 9 weeks than control group. When compared between hPE and Non-hPE group, collagen deposition and fatty change were remarkably decreased in hPE groups, but not in Non-hPE group, in particular, the reduction of collagen deposition was more pronounced in N-hPE than P-hPE group. With analysis of fibrosis grade according to scoring criteria, we could confirm the results above; the score significantly decreased in hPE administration groups (P<0.05; Table 1), and in contrast to 4 rats of grade 4 in Non-hPE group, there were none in both hPE administrated groups. Four rats in N-hPE administrated group were in grade 2, and 3 rats in P-hPE administrated group were in grade 2. Next, we performed zymography analysis to confirm activity of MMPs according to hPE administration in CCl<sub>4</sub>-injured rats. Activity of MMP-9 was highly increased in hPE administration groups, especially in N-hPE group, compared to Non-hPE group, whereas there was no difference in MMP-2 activity (Fig. 3B). These data suggested that hPE administration to CCl<sub>4</sub>-injured rats had efficacy of restoring hepatic fibrosis via reducing abnormal ECM deposition via induction of MMP-9 activation and that N-hPE administration might be more effective than P-hPE administration.



**Fig. 2.** Gene expressions related with liver fibrosis. (A) mRNA expression of Col I was analyzed by RT-PCR. Quantity One program was used for evaluation of band intensity, and values were normalized with 28s rRNA. RNA levels of Col I in N-hPE and P-hPE groups were significantly reduced compared to Non-hPE group. <sup>#</sup>P<0.01 statistically significant compared to CCl<sub>4</sub> group; <sup>\*</sup>P<0.05 statistically significant compared to Non-hPE group. Data were expressed as means ± SD. (B) Protein expression of Col I, α-SMA, and MMP-9 was analyzed by Western blot. An equal concentration of protein of individuals from each of the groups was pooled and a total of 50 µg of protein lysate was loaded onto each well. Actin was used as loading control. The protein levels of Col I and α-SMA were decreased, in contrast the protein levels of MMP-9 were increased in hPE groups compared to Non-hPE group.

**3.4. hPE induced expression of liver regeneration related proteins and promoted proliferation of hepatocyte in CCl<sub>4</sub>-injured rat liver**

Next, we examined whether hPE administration promoted liver regeneration in CCl<sub>4</sub>-injured rat liver model. The levels of IL-6, gp130, ABCG 1, and ABCG2 expression, which are suggested as modulator of liver regeneration [23,24], were increased in both N-hPE and P-hPE administrated groups. In particular, the increase of ABCG2 expression was more pronounced in N-hPE than P-hPE group. Furthermore, both cyclin A and albumin, known as a cell cycle regulator and an indicator of synthetic liver function, respectively increased in hPE administrated groups compared to Non-hPE group with more enhancement in N-hPE group than P-hPE group (Fig. 4A). To evaluate whether hPE



**Fig. 3.** Histopathological analysis to detect collagen deposition and enzyme activity analysis of MMPs. (A) Liver tissue embedded in paraffin was sectioned 5  $\mu$ m, and MT staining was performed. Massive bridging fibrosis and great fatty change occurred after 9 weeks of CCl<sub>4</sub> treatment. Both hPE groups showed decreased deposition of liver collagen as well as low level of bridging fibrosis and fatty change compared to CCl<sub>4</sub> group. By contrast, little change of collagen deposition, bridging fibrosis, and fatty change was observed in Non-hPE group. Scale bar = 100  $\mu$ m. (B) Enzyme activities of MMP-2 and MMP-9 were determined by gelatin zymography analysis. Activities of active MMP-9 were increased in hPE groups, especially in N-hPE group, compared to Non-hPE group.

administration enhance the proliferation of hepatocytes in CCl<sub>4</sub>-injured rat livers, we performed immunohistochemistry with the Ki-67 antibody (Fig. 4B) and presented with the percentage of Ki-67-positive nuclei per total hepatocytes, indicated with Ki-LI (%) (Fig. 4C). In both of hPE group of livers showed increased, nearly 2-fold, percentage of Ki-LI compared to Non-hPE group ( $P < 0.05$ ). These findings suggested that hPE administration on CCl<sub>4</sub>-injured rat liver model could promote liver regeneration through enhancement proliferation activity of hepatocytes, for which N-hPE administration might be more effective than P-hPE administration.

### 3.5. hPE promoted rat hepatic cell regeneration after CCl<sub>4</sub>-injury in vitro

To investigate whether hPE treatment could promote proliferation of injured rat hepatic cells, in vitro proliferation analysis was performed. For construction of hepatocyte injury, 0–15 mM CCl<sub>4</sub> was treated to WB-F344 and T-HSC/Cl-6 cells for 3 h, and viability was measured by MTT analysis (Supplementary Fig. 1). WB-F344 and T-HSC/Cl-6 cells were

cultured in the presence of 0, 40, 80, and 160  $\mu$ g/ml of N-hPE or P-hPE for 24 h after treatment with 6 mM CCl<sub>4</sub>, in which concentration 80% of both cell lines were viable, for 3 h and the proliferation activities were analyzed by BrdU ELISA (Fig. 4D). The proliferation of CCl<sub>4</sub>-injured WB-F344 and T-HSC/Cl-6 cells was significantly enhanced in the presence of 40, 80, and 160  $\mu$ g/ml of N-hPE and P-hPE compared to those cells in the absence of hPE ( $^*P < 0.05$ ). In comparison between N-hPE and P-hPE, the proliferation of WB-F344 was significantly more enhanced with N-hPE of 40, 80 and 160  $\mu$ g/ml than with P-hPE of the same concentration ( $^{\dagger}P < 0.05$ ). Similarly, the proliferation of T-HSC/Cl-6 was significantly more enhanced with N-hPE of 40 and 80 than with P-hPE of the same concentration ( $^{\dagger}P < 0.05$ ). These data demonstrated that hPE treatment promoted the proliferation of CCl<sub>4</sub>-injured rat hepatic cells, for which N-hPE treatment was more effective than P-hPE treatment.

### 3.6. hPE suppressed proliferation of activated T-cells and regulated inflammation related cytokines

Because inflammatory responses were related with progression of liver fibrosis [25], we investigated whether hPE treatment could suppress activated T-cell proliferation and/or regulate inflammation related cytokines. Isolated from UCB and activated by anti-CD3 and anti-CD28, which is T-cell activating mAbs, T-cell proliferation and formation of clusters were significantly suppressed in the presence of 40  $\mu$ g/ml N-hPE at 48 h and 72 h cultivation ( $P < 0.05$ ; Fig. 5A, B). In addition, these suppressive effects were significantly different with dose dependent manner at 0–120  $\mu$ g/ml N-hPE treatment (data not shown). To evaluate which events were regulated by N-hPE treatment for T-cell suppression, multiplex supernatant cytokine assay was performed. As a result, anti-inflammatory cytokines including IL-5, G-CSF, fractalkine,

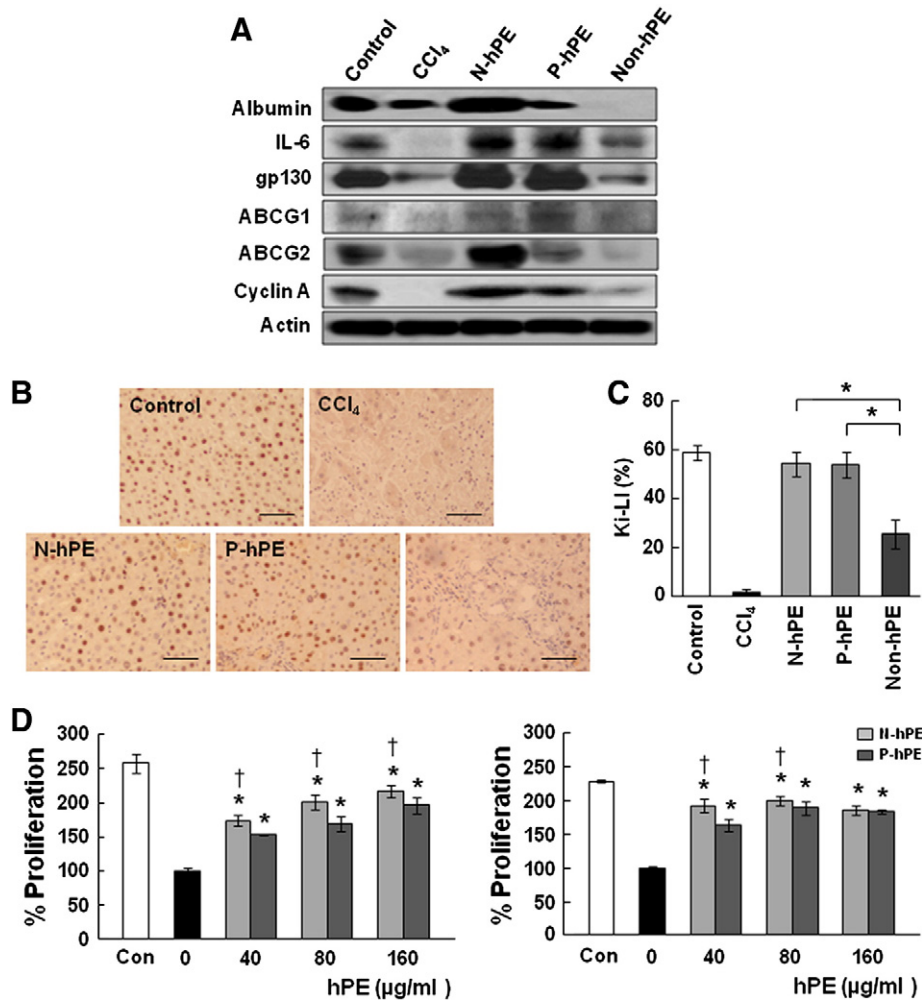
**Table 1**  
Summary of histological grade in each group.

Grade	Control	CCl <sub>4</sub>	N-hPE	P-hPE	Non-hPE
0	6	0	0	0	0
1	0	0	0	0	0
2	0	0	4	3	0
3	0	0	3	4	3
4	0	6	0	0	4
p value			<0.05 <sup>a, b</sup>	<0.05 <sup>a, b</sup>	n.s.

n.s., not significant, comparison with CCl<sub>4</sub> group.

<sup>a</sup> Comparison with non-hPE group.

<sup>b</sup> Comparison with CCl<sub>4</sub> group.



**Fig. 4.** Expression of proteins related with liver regeneration and proliferation activities according to hPEs administration in vivo and in vitro. (A) Expression of albumin and proteins associated with liver regeneration and cycle regulation was more induced in hPE groups than Non-hPE group. Actin was used as loading control. (B) Proliferation assay of rat livers in control, CCl<sub>4</sub>, N-hPE, P-hPE and Non-hPE groups was performed by immunohistochemistry using Ki-67 staining. Scale bar = 100 µm. (C) The percentage of hepatocytes with Ki-67-positive nuclei relative to the total number of hepatocytes of each group was represented as percentage of Ki-67 labeling index (Ki-LI). Data were expressed as means ± SD. \*P < 0.05 compared to Non-hPE group. (D) Proliferation assay of WB-F344 (left) and T-HSC/Cl-6 cells (right) treated with 0–160 µg/ml of N-hPE and P-hPE for 24 h after 6 mM CCl<sub>4</sub> treatment for 3 h at 37 °C was performed using BrdU ELISA. Data were expressed as means ± SD. \*P < 0.05 compared to no treatment of hPE; †P < 0.05 indicates significant differences between N-hPE and P-hPE group.

IL-10 and IL-13 were elevated (Fig. 5C), and pro-inflammatory cytokines, such as interferon-γ (IFN-γ), IL-1β and IL-2 were decreased in activated T-cell culture supernatant with presence of 40 µg/ml N-hPE for 72 h (Fig. 5D). Tumor necrosis factor-α (TNF-α) was decreased, and IL-6 was increased in T-cell culture supernatant with presence of 40 µg/ml N-hPE for 72 h (Fig. 5D). These data suggested that N-hPE had suppressive effects to T-cells via regulation of inflammation related cytokines.

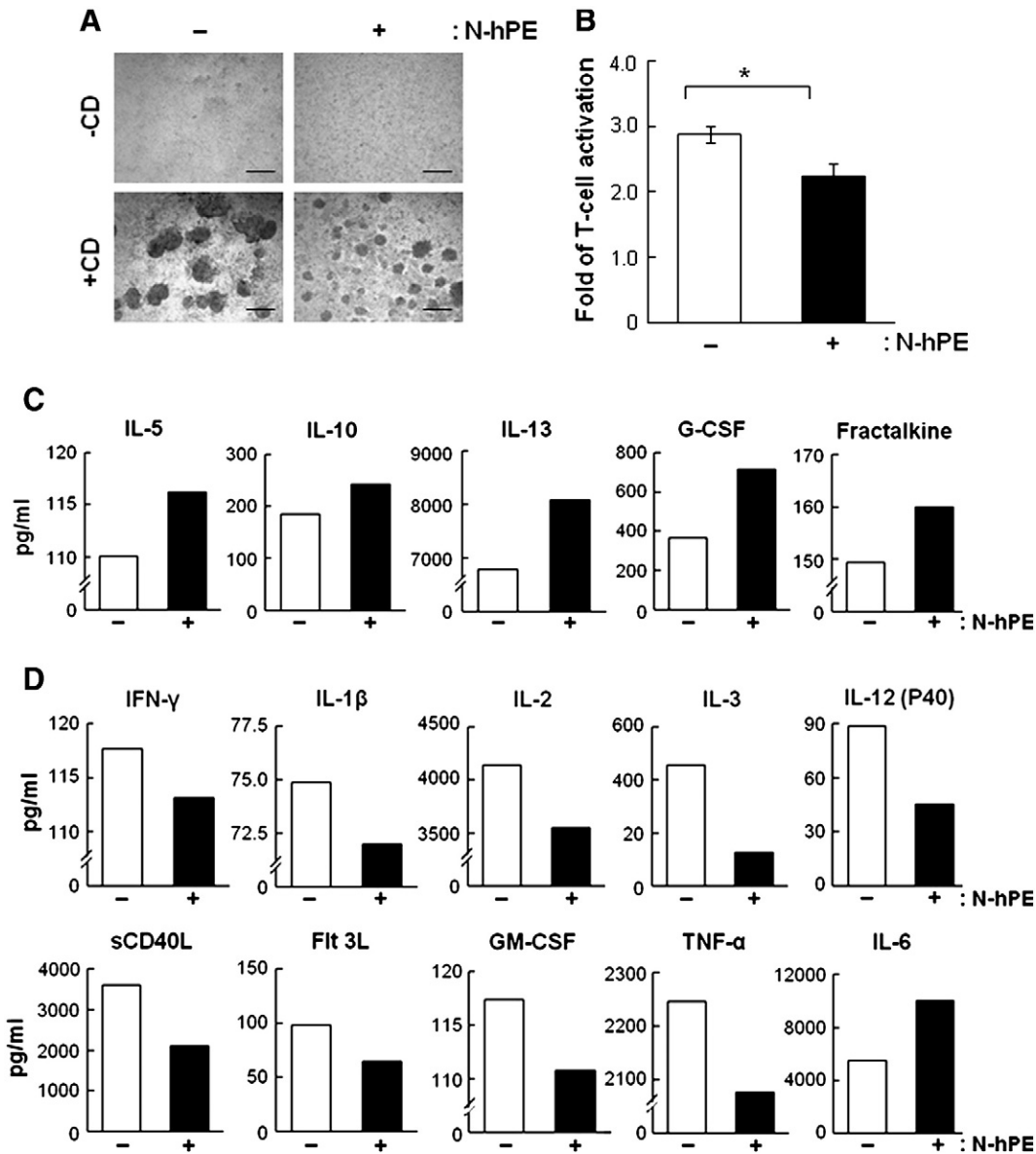
#### 4. Discussion

This is the first report on usefulness of hPE treatment for the therapeutic purpose in liver disease model with molecular mechanisms of anti-fibrosis, anti-inflammation and liver regeneration in the present study.

In development of cirrhosis, there is a common pathogenesis that chronic hepatic injury with inflammation results in fibrosis and loss of hepatocytes, and accumulation of such injury ultimately leads to cirrhosis regardless of the origin of injury [25,26]. In order to prevent and restore the process of cirrhosis, there have been studies of specific anti-fibrotic treatment (e.g. angiotensin II antagonist, IFN-γ, peroxisomal proliferator-activated receptor γ ligands, and colchicine) for specific

target (e.g. stellate cell activation, fibrogenesis, and oxidative stress). However, no consistent efficacy has been clearly demonstrated, and many of these specific compounds have adverse effects on collateral cells or organs outside the fibrogenic responses [26]. Regarding natural components, although there are reports in animal models that injured liver could be repaired [26–29], they might harbor significant toxicities including hepatotoxicity itself that needs to be overcome for clinical applications [30,31]. Therefore, hPE has been suggested as a promising therapeutic agent for liver disease with growing evidences that hPE is an abundant reservoir of many useful bioactive molecules [5–7] and that hPE has effects on anti-fibrosis, inflammation regulation [32], anti-oxidation [12,33], and liver regeneration [15]. Also, hPE has been shown to promote potentials for hepatogenic differentiation in placenta-derived stem cells [34].

Several bioactive molecules in hPE have been spotlighted in the Western medicine as well as in the Oriental medicine [5,10,11]. Because the placenta supports fetal development through the synthesis of various molecules during pregnancy [35], there are abundant biologically important factors, and some of these cytokines and growth factors are known to be essential for liver regeneration [5–7,9]. In addition, it has been reported that placenta extract stimulate tissue repair process [15,36], has therapeutic effects in chronic non-healing wounds [8,37],



**Fig. 5.** Immunomodulatory effects of hPE. Mononuclear cells isolated from UCB were cultured in the absence or presence of 40 μg/ml N-hPE with or without 1 μg/ml anti-CD3 and anti-CD28, which are T-cell activating mAbs, for 48 h and 72 h. (A) T-cell clusters resulting from activation of clonal expansion were shown in bright field at 72 h cultivation. N-hPE suppressed clonal expansion of activated T-cells. Scale bar = 50 μm. (B) Proliferation activity of T-cells was determined by BrdU ELISA analysis at 48 h cultivation. Proliferation of activated T-cells was significantly decreased with N-hPE treatment. \* $P < 0.05$  compared to no treatment of hPE. (C,D) Supernatant from activated T-cell culture with or without 40 μg/ml N-hPE for 72 h was analyzed with multiplex supernatant cytokine assay. N-hPE altered profiles of systemic inflammatory cytokines.

and has anti-inflammatory effects [32]. However, despite identification of biologically active molecules and trials for several diseases, precise underlying mechanisms still remain largely unknown and warrant further investigation [5,7,38,39].

Molecular activities of placental extract may vary according to manufacturing processes. For instance, excessive digestion steps, organic phase extraction steps, and especially heating steps can affect the efficacy of functional molecules derived from the placenta. Although there are already several methods known for placenta extraction, loss of many valuable biologically active molecules is inevitable during the steps in which heating and pressure are applied. Moreover, during the purification procedure for a single active molecule, other useful factors as well as the purifying methods, such as UV analysis, diphenylamine technique and gel electrophoresis, have been indicated the problem of quality control and unsuitable industrial application [5]. Furthermore, previous studies on placenta extraction methods have been largely focused on the effective way of

purification of target molecules [5,12] with less interest in methods to preserve useful components or in comparison of efficacy between those methods. Therefore, we investigated on the method of placenta extraction that could preserve biologically active components showing beneficial effects in animal model and demonstrate thereby that hPE has therapeutic effects on  $\text{CCl}_4$ -injured rat model and in vitro model as well as that hPE has function on regulation of activated T-cell proliferation and inflammatory cytokines. Multiplex supernatant cytokine assay showed that a number of cytokines and growth factors were contained in hPE and that markedly different concentrations of several cytokines and growth factors were observed in N-hPE and P-hPE. The levels of some hematopoietic cell proliferation stimulators, inflammation regulatory cytokines, and growth factors including GM-CSF, fractalkine, IFN $\alpha$ 2a, RANTES, and PDGF were higher in N-hPE than in P-hPE [34]. These results suggest that active biological factors are contained more in N-hPE because the process of denaturation through pasteurization is absent.

Next, to investigate whether N-hPE and P-hPE could improve liver injury and whether they had different efficacy, blood chemistry analysis and ICG uptake/excretion assay were performed. Serum levels of GOT/AST and GPT/ALT were reduced significantly in hPE administrated groups compared to Non-hPE administrated group, which are in agreement with previous reports [15]. Although serum levels of albumin and total bilirubin were not significantly different (data not shown), the protein level of albumin was enhanced in liver of hPE group with more pronouncement in N-hPE group, whereas it was scarcely detected in Non-hPE group. These findings may reflect that 3 weeks is enough to detect albumin production in liver tissue, while not sufficient to detect in serum in this system. For liver function test, we used ICG uptake/exclusion assay, which is the only agent approved by the United States Food and Drug Administration for clinical application in liver function assessment and which is being used both experimentally in animal model and clinically in general [17,40]. The results of ICG uptake/excretion were significantly induced in hPE group compared to Non-hPE group in CCl<sub>4</sub>-injured rats showing the liver function improved by hPE administration.

On the other hand, fibrosis-causing gene and proteins were reduced in hPE groups and treatment of hPE reduced the level of TNF- $\alpha$ , which has a direct stimulatory effect on collagen synthesis [25], in T-cell culture supernatant. These results suggest that hPE reduced expression of fibrosis-related factors as well as collagen synthesis stimulator produced by active T-cells, and consequently led to reduction of abnormal ECM structure in CCl<sub>4</sub>-injured rat liver as shown in immunohistological data with anti-fibrotic effect. Generally, it is a well known correlation between a balance for the expressions of MMPs or extracellular matrixes and accumulation of collagen in damaged tissues. Recently, Li and their colleagues reported that low anticoagulant activity heparin (LAAH) can decrease rat hepatic fibrosis induced by either CCl<sub>4</sub> or porcine serum injection via extracellular signal-regulated kinase signal transduction pathway and activator protein-1 activity in activated hepatic stellate cells when LAAH are administered for a long time into rat model [41]. However, the precise mechanism is still unknown. Many kinds of cytokines might be involved in these repair systems. Due to the fact that hPE is rich in several cytokines and bioactive molecules [16], hPE could trigger mechanisms for repair via various signal pathways. Therefore, we should be able to study these mechanisms including anti-fibrosis to evaluate the detailed effects of hPE in the future.

In addition, we investigated whether N-hPE and P-hPE administration could promote liver regeneration in vivo and hepatic cell proliferation in vitro and whether they had different efficacy. The expression of proteins, which are essential for liver regeneration such as IL-6 and gp130, was dramatically induced in both hPE groups, in particular, expression of albumin, ABCG2, and cyclin A was more increased in the N-hPE group than in the P-hPE group. These data are in agreement with previous reports indicating that the expression of IL-6 and gp-130 enhances liver regeneration [23,24,42,43]. Furthermore the proliferation activity, which was indicated with the percentage of Ki-67 positive hepatocytes, was significantly enhanced in hPE groups, showing similar percentages with control rat livers. We also confirmed that hPE treatment in vitro promoted proliferation of hepatic cells after CCl<sub>4</sub>-injury at dose dependent manner and that N-hPE treatment enhanced proliferation activity more than P-hPE treatment on both rat hepatic epithelial stem-like cells and rat hepatic stellate cells. Although, hepatic stellate cells are known to contribute to fibrosis in injured liver [44], multiple functions of stellate cells in liver regeneration have been reported that they act as undifferentiated cells with stem cell characteristics, which might play an important role in liver regeneration [45,46]. Therefore, enhanced proliferation of hepatic stellate cells by hPE treatment in our data might have association with activation of liver regeneration, and this hypothesis needs to be further verified.

With regard to cytokines, hPE treatment induced the level of IL-6, which is known to have dual functions for liver regeneration and pro-

inflammation, in T-cell culture supernatant in our study. These results may explain that hPE can trigger regeneration of damaged liver via acceleration of hepatic cell proliferation and induction of factors related to liver regeneration such as IL-6 by active T-cells. In addition, N-hPE and P-hPE showed differences in the degree of effects, which suggests N-hPE might be more effective to induce activities of molecules related with regeneration in CCl<sub>4</sub>-injured rat liver model because the preparation method of hPE using pasteurization or not resulted in the difference of components in hPE.

Inflammatory responses are related with the progression of liver fibrosis [25], and it is necessary to take anti-inflammatory measures in subjects with liver fibrosis. The placenta as a fetomaternal organ has abundant immune-regulating cytokines with effects on immune modulation [34,47,48]. Thus, we investigated whether hPE treatment could suppress activated T-cell proliferation and/or regulate inflammation related cytokines. In our study, hPE treatment suppressed T-cell activation and altered systemic inflammatory cytokine profiles in vitro culture system. These data are in accord with previous reports that liver regeneration [49] and expression of IL-10, which is an important cytokine for reduction of liver fibrosis [50], were enhanced and that expression of IFN- $\gamma$  and TNF- $\alpha$ , which is crucial for promotion of liver regeneration was suppressed [51,52]. It suggests that hPE has a potential for the prevention of liver fibrosis progressed by inflammation and for promotion of liver regeneration.

In conclusion, hPE may be useful for the treatment of liver injury through restoration of liver function, reduction of fibrosis and inflammation, and induction of liver regenerative effects. Our study not only provides further understanding of the potential of hPE in liver diseases, but also contributes to the foundation for a potential new treatment for liver diseases.

Supplementary materials related to this article can be found online at [doi:10.1016/j.intimp.2011.02.012](https://doi.org/10.1016/j.intimp.2011.02.012).

## Acknowledgments

We thank Kyung-Seon Shin and Ji-Ye Song for technical supports of animal experiments. This work was supported by the Korea Healthcare Technology R&D Project, Ministry for Health Welfare & Family Affairs, Republic of Korea (A084633).

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