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By Chanon Ngamsombat, Walailak Chaiyasoot

ORIGINAL ARTICLE

184 **Curcumin Attenuates Hydrogen Peroxide-Induced Cytotoxicity in Human Neuroblastoma SK-N-SH Cells**
Rungtip Soi-ampornkul, et al.

191 **Low Complication Rate Associated with Total Laparoscopic Hysterectomies Using the Retroperitoneal Approach: A Series of 1,092 Cases in Siriraj Hospital**
Pisutt Srichaikul, et al.

198 **Transarterial Embolization: Alternative Treatment of Early and Late Postpartum Hemorrhage at a Single Tertiary Center**
Satit Rojwatcharapibarn, et al.

204 **Serum Neuropeptide Y and Leptin Levels compared between Non-pregnant and Pregnant Women in Overall, Non-obese, and Obese Subjects**
Chantacha Sitticharoon, et al.

213 **Additional Hyaluronic Acid Binding Selection Decreased Sperm DNA Damage after Conventional Semen Preparation in Infertile Patient with Abnormal Semen Analysis**
Roungsin Choavaratana, et al.

219 **Serum Adiponectin, Visfatin, and Omentin Compared Between Non-Pregnant and Pregnant Women in Overall, Non-obese, and Obese subjects**
Chantacha Sitticharoon, et al

227 **Ultrasonication Extraction and Solid Phase Extraction Clean-up for Quantification of Aristolochic acid I in the Thai Herbal Antitussive 3 Formula by High Performance Liquid Chromatography with Photodiode Array Detection**
Jantanee Wattanarangsan, et al.

233 **Obstetric and Perinatal Outcomes of Singleton Pregnancies after In-Vitro Fertilization in Siriraj Hospital, A Matched Case-Control Study**
Roungsin Choavaratana, et al.

238 **Development of a Novel Method for Determination of 11 Antiretroviral Concentrations in Human Plasma by Ultra-Performance Liquid Chromatography-Photodiode Array Detection Method**
Rasda Boonprasert, et al.

247 **Analysis of Extrahepatic Collateral Arteries in Transcatheter Arterial Chemoembolization of Hepatocellular Carcinoma**
Chanon Ngamsombat, Walailak Chaiyasoot

CASE REPORT

254 **Metastatic Malignant Melanoma of the Urinary Bladder: A Case Report and Review of Literature**
Chaiyong Nualyong, et al.

REVIEW ARTICLE

260 **Unusual Pediatric Abdominal Cysts: A Pictorial Review of Imaging Findings**
Pira Neungton, et al.

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Curcumin Attenuates Hydrogen Peroxide-Induced Cytotoxicity in Human Neuroblastoma SK-N-SH Cells

Rungtip Soi-ampornkul, Ph.D.*, Sandeepa Ghimire, B.Sc.*, Wipawan Thangnipon, Ph.D., Nirut Suwanna, D.V.M., Ph.D.***, Chinnavuth Vatanashevanopakorn, M.D., Ph.D.***

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ABSTRACT

Objective: Cellular damage induced by oxidative stress has been involved in the development of neurodegeneration. Curcumin, a dietary polyphenol found in the rhizome of *Curcuma longa*, has been shown, both *in vitro* and *in vivo*, to be an effective reactive oxygen scavenging molecule. We investigated an anti-oxidative effect of curcumin against H_2O_2-induced toxicity in human neuroblastoma cell line SK-N-SH.

Methods: The SK-N-SH cells were pre-treated with curcumin 2 hours prior to H_2O_2 treatment. We measured cell viability, intracellular reactive oxygen species (ROS) levels, expression of apoptotic-related proteins and caspase-3 activity 24 hours post H_2O_2-induced cytoxicity.

Results: Treatment with curcumin at concentrations ranging from 5 to 50 µg/mL was not cytotoxic. Pre-treatment with curcumin at the concentrations of 5 to 50 µg/mL prior to H_2O_2 exposure caused a significant decrease in intracellular ROS levels and a significant increase in cell viability in a dose-dependent manner. Expression of activated form of caspase-3 and BAX, a pro-apoptotic protein, measured by Western blotting were reduced when the SK-N-SH cell line was pre-incubated with curcumin. The curcumin pre-treated cells also exhibited less caspase-3 activity.

Conclusion: Curcumin has protective effects against H_2O_2-induced toxicity in a dose-dependent manner through its anti-apoptotic and anti-oxidative properties in an *in vitro* H_2O_2-treated SK-N-SH model.

Keywords: Curcumin; apoptosis; anti-oxidant; oxidative stress; SK-N-SH cells (Siriraj Med J 2018;70: 184-190)

INTRODUCTION

Oxidative stress-mediated cellular damage has long been recognized to be a crucial factor in pathophysiology of several neurological disorders, for instance, Alzheimer's disease, stroke and Parkinson's disease.[1-3] The damage is a result of an imbalance between anti-oxidants and pro-oxidants, which are known as reactive oxygen species (ROS). Super-oxide radical (O_2-) and hydrogen peroxide (H_2O_2) are examples of ROS. Excess ROS reacts with DNA, lipids and proteins leading to DNA damage, lipid peroxidation and protein oxidation, respectively. Consequently, cell death via apoptosis is unavoidable.[4-5]

Curcumin (diferuloylmethane), the most common form of curcuminoids found in the rhizome of *Curcuma longa* or turmeric, has long been commonly used for culinary purposes.[6-8] Beneficial biological properties of curcumin include anti-neoplastic, anti-inflammation, anti-oxidative, and anti-angiogenic properties.[6-9] Among these biological properties, its potent anti-oxidative effect is well known. Several investigators have reported the protective effects of curcumin against oxidative stress both *in vitro* and *in vivo*. Curcumin reduces rat pheochomocytoma PC12 cell death induced by β-amyloid via a reduction of oxidative stress and DNA damage.[10] In the *in vitro* model of mouse neuroblastoma Neuro-2A cell line, curcumin protects the cells against H_2O_2- induced oxidative stress as determined by a decrease in intracellular ROS and apoptosis.[11] These studies indicated that curcumin could

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be used for treatment or prevention of oxidative stress-related diseases. Here we used H_2O_2-induced cytotoxicity model in human neuroblastoma cell line, the SK-N-SH, to investigate the neuroprotective activity of curcumin.

MATERIALS AND METHODS

Chemicals

Curcumin was purchased from Government Pharmaceutical Organization (GPO), Bangkok, Thailand. Dimethyl sulfoxide (DMSO), H_2O_2 and DCFH-DA were from Sigma. Apo-ONE® homogeneous caspase-3/-7 assay and CellTiter-Blue® cell viability assay were obtained from Promega. Clarity™ Western ECL Blotting substrate kits were purchased from Bio-Rad. Stock solution of curcumin was prepared by dissolving curcumin powder in DMSO at a concentration of 10 mg/mL. Stock curcumin was kept at -20°C until diluted before used.

Cell culture and treatment of curcumin/H_2O_2

The human neuroblastoma cell line SK-N-SH was purchased from the ATCC (Rockville) and cultivated in complete fibroblast medium (DMEM with 2 mM L-glutamine, 10% v/v fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 100 units/mL penicillin and 100 µg/mL streptomycin, and all were from Gibco) in a 37°C incubator with 5% CO_2. Passage numbers of the SK-N-SH cells used in this study were between 9 and 39. Cells were maintained for 24 hours before an addition of curcumin at seven different concentrations (5, 10, 15, 20, 25, 50 and 100 µg/mL) for 2 hours. The cells were then treated with 300 µM H_2O_2 and incubated for 24 hours. The control cells were cultured in complete fibroblast medium without $H_2O_2$and curcumin treatment.

Cell viability

Measurement of cell viability was performed by CellTiter-Blue® cell viability assay. In brief, 25,000 cells were cultured in each well of a 96-well plate and incubated overnight. Curcumin/H_2O_2 treatment was done as described. Twenty four hours after H_2O_2 treatment, the medium was then carefully discarded and replaced with 100 µL of fresh complete fibroblast medium. 10 µL CellTiter-Blue® reagent was then added into each well. The SK-N-SH cells were kept in a 37˚C incubator with 5% CO_2 for an hour. Fluorescence was measured with a microplate reader (Synergy HT, BioTek Instrument) using 540 nm and 590 nm as the excitation and emission wavelength, respectively. The fluorescent signal was directly proportional to the percentage of cell viability.

Intracellular ROS production

25,000 cells were cultured in each well of a 96-well plate and incubated overnight at 37°C with 5% CO_2. Curcumin/H_2O_2 treatment was done as described. 24 hours after H_2O_2 treatment, 5 µL DCFH-DA (a final concentration of 50 µM) was added to each well and incubated for 1 hour, and fluorescence intensity was measured by microplate reader (Synergy HT, BioTek Instrument) using 485 nm and 530 nm as the excitation and emission wavelength, respectively. The fluorescence intensity was proportional to the levels of ROS produced.

Assessment of DNA fragmentation by gel electrophoresis

To determine DNA fragmentation in apoptotic cells, 1 x 10^6 cells were cultured overnight in each well of a 6-well plate. Curcumin/H_2O_2 treatment was done as described. After 24 hours of incubation, cells were collected and isolation of genomic DNA was done with genomic DNA mini kit (Geneaid) as per the manufacturer's instruction. Extracted genomic DNA was subjected to gel electrophoresis, and the image was obtained with GelDoc™ EQ (Bio-Rad).

Western blot analysis

1 x 10^6 cells were cultured overnight in each well of a 6-well plate. Curcumin/H_2O_2 treatment was done as described. Twenty four hours after H_2O_2 treatment, medium was then carefully removed and washed once with fresh medium. Cells were lysed in a lysis buffer. After centrifugation of the lysate, protein concentrations were measured by Nanophotometer (Implen). 10% SDS-PAGE was then done with 30 µg protein samples. Proteins were blotted onto a polyvinylidede fluoride membrane (Bio-Rad). Membranes were blocked with 5% non-fat dried milk in TBS for 1 hour before incubating with primary antibodies (rabbit anti-human actin antibody at 1:1,000 dilution, Cell Signaling; rabbit anti-activated human caspase-3 antibody at 1:500 dilution, Cell Signaling; mouse anti-human BAX antibody at 1:500 dilution, Santa Cruz) in 0.1% Tween-20 in TBS at 4°C overnight. The membrane was incubated with secondary antibodies, conjugated with horseradish peroxidase (HRP), against rabbit or mouse (1:1,000, both from Cell Signaling) at room temperature for 1 hour. Membranes were then incubated with Clarity™ Western ECL Blotting substrate (Bio-Rad). Blots were visualized by ImageQuant LAS 4010 (GE Healthcare). Density of bands was quantified using densitometric analysis of ImageJ software (National Institutes of Health).

Measurement of caspase-3/-7 activity

25,000 cells were cultured in each well of a 96-well plate and incubated overnight at 37˚C with 5% CO_2. Curcumin/H_2O_2 treatment was done as described. After 24 hours of incubation, medium was discarded and 100 µL of fresh complete fibroblast medium was added into each well. Activity of caspase-3 was assessed by Apo-ONE® homogeneous caspase-3/-7 assay as per manufacturer's instructions. Briefly, 50 µL caspase-3/-7 reagent was added into the culture and incubated for 1 hour at 37°C and 5% CO_2. Fluorescent signal was then obtained at 521 nm with micro-plate reader (Synergy HT, BioTek Instrument) with the excitation wavelength of 499 nm.

Statistical analysis

Data is presented as the mean ± SEM (n=3). Statistical analysis was by one-way ANOVA, followed by Dennett's test for comparing between control/H_2O_2-treated and curcumin pre-treated experimental groups which was done with Graphpad Prism version 5 (GraphPad Software). P-value of less than 0.05 was used for determining significant differences.

RESULTS

Cytotoxicity of curcumin on the SK-N-SH cells

We investigated whether curcumin is toxic to the SK-N-SH cells by treating the cells with different concentrations of curcumin (5-100 µg/mL) for 24 hours. Cell viability after treatment with curcumin at the concentration of 5, 10, 15, 20, 25, 50 and 100 µg/mL were 99.1±2.23%, 95.13±2.08%, 97.81±1.37%, 98.33±0.53%, 97.43±2.25%, 94.7±3.35% and 38.88±10.98%, respectively (Fig 1A). The data showed that exposure to curcumin at the concentrations ranging from 5 to 50 µg/mL for 24 hours did not alter cell viability. However, curcumin was toxic at the concentration of 100 µg/mL as demonstrated by a significant reduction of cell viability (one-way ANOVA: F=23.34, p<0.0001; Dunnett's post-hoc test: p<0.0001 control vs 100 µg/mL curcumin; n=3 for each group). The cytotoxic concentration of curcumin that caused 50% reduction of cell viability (CC50) was 92.045 µg/mL (Fig 1B).

Curcumin protects SK-N-SH cells against H_2O_2-induced toxicity

To test whether curcumin exhibits protective effect against H_2O_2-induced cytotoxicity, we first identified the H_2O_2 concentration that could reduce cell viability by 50% by treating the SK-N-SH cells with various concentrations

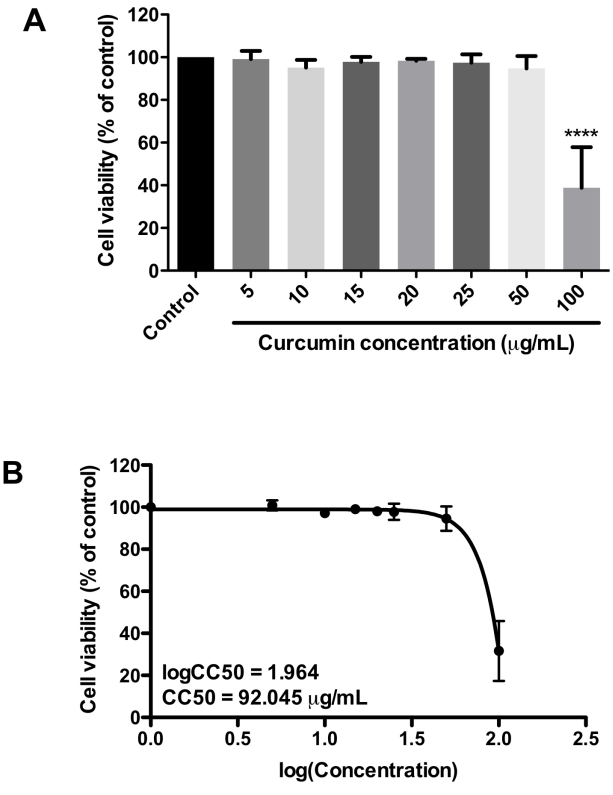


Fig 1. The effect of curcumin on cell viability. Cells were incubated at 37°C with several concentrations of curcumin (5, 10, 15, 20, 25, 50 and 100 µg/mL) for 24 hours. The cytotoxicity of curcumin was measured using CellTiter-Blue® reagent. ****p<0.0001 vs control group. Values are mean±S.E.M. from three independent experiments.

of H_2O_2. Treatment of H_2O_2 at all tested concentrations showed significantly decreased cell viability (one-way ANOVA: F=68.68, p<0.0001; Dunnett's post-hoc test: p<0.0001 control vs all H_2O_2 concentrations; n=3 for each group). The reduction of cell viability was in a dose-dependent manner (Table 1 and Fig 2A). From the data, we chose 300 µM H_2O_2, which induced approximately 50% reduction in cell viability, for assessing the protective effect of curcumin.

We then pre-treated the SK-N-SH with different concentrations of curcumin (5, 10, 15, 20, 25, 50 and 100 µg/mL) 2 hours prior to H_2O_2 treatment at a concentration of 300 µM. We found a significant increase in cell viability in curcumin pre-treated cells at all concentrations except 100 µg/mL as compared to the H_2O_2 treated group (Fig 2B, one-way ANOVA: F=30.15, p<0.0001: Dunnett's post-hoc test: p<0.001 300 µM H_2O_2 vs 5, 10, 15, 20, 25 and 50 µg/mL curcumin; n=3 for each group). The data indicated that pre-treatment with curcumin at the concentrations rangilng from 5 to 50 µg/mL rescued H_2O_2-induced cytotoxicity.

TABLE 1. Concentration-dependent cytotoxicity of H_2O_2 in SK-N-SH cells.

H_2O_2 concentration (μM)	Cell viability (% of control)
37.5	62.87±2.17%
75	59.60±3.95%
150	56.64±1.92%
300	52.48±1.21%
600	49.28±1.32%
1,200	37.4±4.48%
2,400	25.56±2.27%

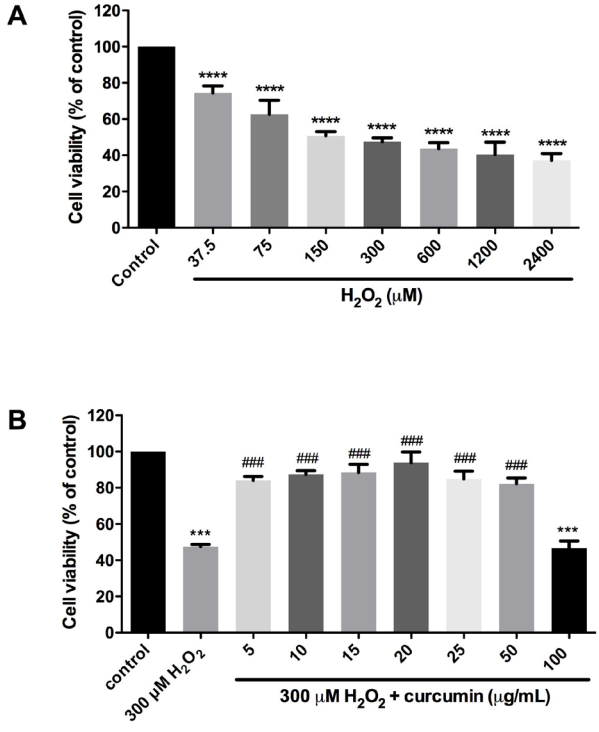


Fig 2. Effect of H_2O_2 on cells viability. (A) Cells were treated with different concentrationsof H_2O_2 (37.5, 75, 150, 300, 600, 1,200 and 2400 μM) for 24 hours. Percentage of cell viability was inversely correlated with H_2O_2 concentrations. (B) Protective effect of curcumin on H_2O_2- induced cytotoxicity was demonstrated at 5, 10, 15, 20, 25 and 50 μg/mL concentrations. Pre-treatment of curcumin at 100 μg/mL did not rescue cytotoxicity induced by H_2O_2. ***p<0.001 and ****p<0.0001 compared to the control group, ###p<0.0001 compared to the H_2O_2-treated group. Values are mean±S.E.M. from three independent experiments.

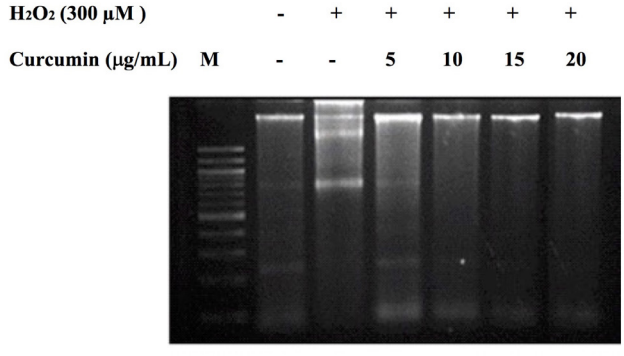


Fig 3. Protective effect of curcumin (5, 10, 15 and 20 μg/mL) on H_2O_2-induced apoptotic DNA fragmentation in SK-N-SH cells. M; DNA ladder.

Curcumin attenuates H_2O_2-induced apoptosis in SK-N-SH cells

DNA fragmentation is one of the observed features in apoptotic cells. If curcumin could potentially inhibit apoptosis in H_2O_2-treated cells, reduction of DNA fragmentation would be evident in cells pre-treated with curcumin prior to H_2O_2 addition. We, thus, performed a gel electrophoresis to see whether the DNA fragmentation could be ameliorated by curcumin (Fig 3). Cells treated with 300 μM H_2O_2 showed a more intense smear pattern compared to the control group. Interestingly, pre-treatment with curcumin at the concentration of 5, 10, 15 and 20 μg/mL dose-dependently decreased the smear. The findings support that H_2O_2-treated cells underwent apoptosis and this could be prevented by pre-treatment with curcumin.

To further investigate the anti-apoptotic property of curcumin, we checked the expression levels of apoptotic-related proteins. Western blotting demonstrated that H_2O_2treatment triggered an up-regulation of the pro-apoptotic protein BAX to be 166.45±11.99% of the control (Fig 4A; one-way ANOVA: F=16.99, p=0.0002; Dunnett's post-hoc test: p<0.001; n=3 for each group). Interestingly, pre-treatment of curcumin at the concentration of 5,

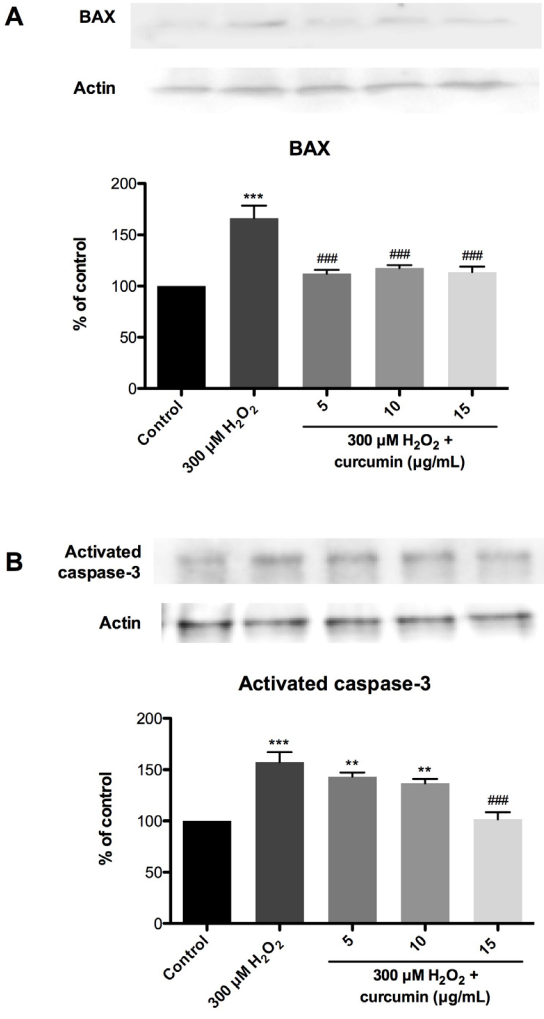


Fig 4. Pre-treatment of curcumin decreased levels of pro-apoptotic protein BAX (A) and activated caspase-3 (B). The SK-N-SH cells were pre-treated with 5, 10 or 15 μg/mL curcumin for 2 hours before $H_2O_2$was being added. Western blot analysis of samples 24 hours after H_2O_2treatment showed a significant decrease in BAX levels when the cells were pre-treated with curcumin at all concentrations. Activated caspase-3 levels were downregulated only when cells were pretreated with 15 μg/mL curcumin. **p<0.01 and ***p<0.001 compared to the control group, ###p< 0.001 compared to the H_2O_2-treated group. Values are mean ± S.E.M. from three independent experiments.

10 and 15 μg/mL prior to H_2O_2 addition effectively reversed the increase in BAX expression induced by H_2O_2 (Dunnett's post-hoc test: p<0.001; n=3 for each group). Likewise, H_2O_2 treatment induced a significant increase in activated caspase-3 levels compared to control (157.41±9.66%; Fig 4B; one-way ANOVA: F=19.05, p=0.0001; Dunnett's post-hoc test: p<0.001; n=3 for each group). The increase in activated caspase-3 levels mediated by H_2O_2 was inhibited by pre-treatment of curcumin at the concentration of 15 μg/mL (Dunnett's post-hoc test: p<0.001; n=3 for each group).

In addition, we checked whether a reduction in activated caspase-3 levels by curcumin resulted in a

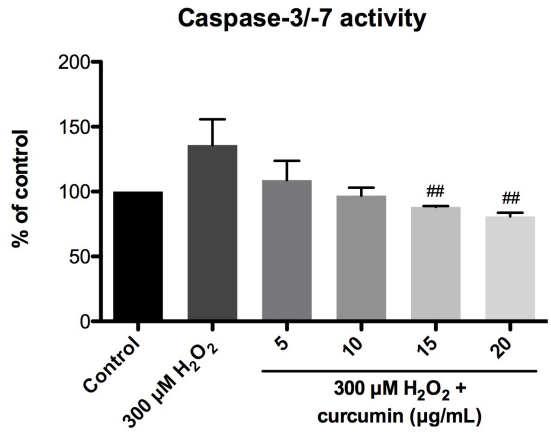


Fig 5. Reduction of caspase-3/-7 activity in samples pre-treated with curcumin. SK-N-SH cells were pre-treated with curcumin (5, 10, 15 and 20 μg/mL) prior to inducing apoptosis by H_2O_2. Inhibition of apoptosis by curcumin was demonstrated by a reduction in caspase-3/-7 activity in samples pre-treated with 15 and 20 μg/mL curcumin. ##p< 0.01 compared to the H_2O_2-treated group. Values are mean ± S.E.M. from three independent experiments.

decrease in H_2O_2-induced apoptosis. Thus, the activity of caspase-3/-7 was assessed by Apo-ONE® homogeneous caspase-3/-7 assay. We found that H_2O_2 treatment caused a rise in caspase-3/-7 activity (135.84±19.85%; Fig 5). Pre-treatment of curcumin at the concentrations of 15 and 20 μg/mL prior to an addition of H_2O_2 significantly reduced caspase-3/-7 activity to 88.17±0.78% and 80.83±2.96%, respectively (Fig 5, one-way ANOVA: F=3.379, p=0.039; Dunnett's post-hoc test: p<0.01; n=3 for each group). All of the data demonstrated that pre-treatment of curcumin reduced H_2O_2-induced apoptosis in SK-N-SK cells.

ROS induction by H_2O_2 can be scavenged by curcumin

To determine whether anti-apoptotic property of curcumin is mediated via a reduction in ROS levels, we measured intracellular ROS levels by DCFH-DA assay. We found that the percentage of ROS levels when the cells were pre-treated with curcumin was significantly decreased compared to H_2O_2-treated group (Fig 6, one-way ANOVA: F=32.1, p<0.0001; Dunnett's multiple comparison test: p<0.001; n=3 for each group). The results suggested that the anti-oxidative property of curcumin contributed, in part, to its anti-apoptotic property.

DISCUSSION

Oxidative stress is caused by excessive levels of ROS. Excess ROS leads to damages of cellular components including lipids, proteins and DNAs. Consequently, the damages contribute to pathogenesis of many age-associated and neurodegenerative diseases. Among a great variety of ROS, H_2O_2 is a major ROS found since it is a

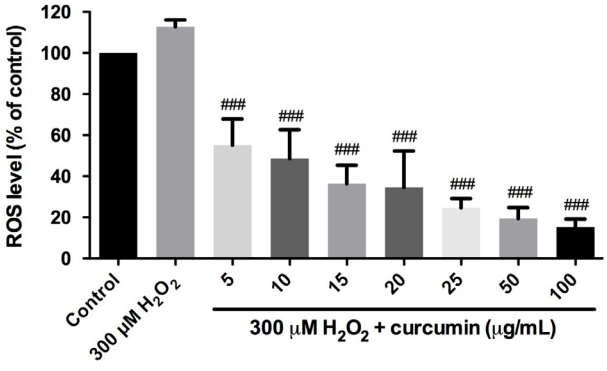


Fig 6. Intracellular ROS levels were reduced in samples pre-treated with curcumin. Cells were pre-treated with various concentrations of curcumin (5, 10, 15, 20, 25, 50 and 100 μg/mL) 2 hours prior to H_2O_2 addition. ROS levels were measured by DCFH-DA assay 24 hours after H_2O_2-induced cytotoxicity. ###p<0.001 as compared to the H_2O_2-treated group. Values are mean ± S.E.M. from three independent experiments.

by-product of aerobic metabolism in mitochondria and peroxisome.[5,12] Oxidative damage by H_2O_2 is normally prevented by catalase, an enzyme that converts H_2O_2 to water and oxygen.[5]

Curcumin has long been shown to exhibit a wide range of pharmacological activities, such as anti-inflamation,[13] anti-oxidant[10-11] and anti-cancer.[14] Several *in vitro* studies reported protective effects of curcumin against oxidative agents in various cell types. For instance, curcumin at a concentration of 10 μg/mL significantly reduced oxidative stress triggered by beta-amyloid in the rat pheochromocytoma PC12 cell line and led to an increase of cell viability.[10] An increase in cell viability and an attenuation of apoptosis, a consequence of intracellular ROS reduction, were found when the H_2O_2-treated mouse neuroblastoma cell line Neuro-2A was pre-treated with curcumin at 20 and 25 μg/mL.[11] Moreover, simultaneously addition of 25-100 μM curcumin and H_2O_2 protected H_2O_2-induced cell damage in the ng108-15 cell culture, a hybrid line between mouse neuroblastoma and rat glioma.[15] In our study, we assessed the protective effect of curcumin on H_2O_2-induced cytotoxicity in the human neuroblastoma SK-N-SH cell line. Our studies demonstrated that treating the cells with H_2O_2 caused a reduction in cell viability in a dose-dependent manner. This reduction can be significantly ameliorated by pre-treating the cells with curcumin at six different concentrations (5, 10, 15, 20, 25 and 50 μg/mL). Additional data revealed that pre-treatment of curcumin at the concentration of 5, 10, 15, 20, 25, 50 and 100 μg/mL prior to cytotoxicity induction by H_2O_2 led to a significant decrease in intracellular ROS levels. Our data was consistent with other studies,[10-11] which showed that pre-treatment of curcumin at 10 μg/mL,[10] 20 μg/mL[11] and 25 μg/mL[11] prior to an addition of oxidative agents into rodent neural lineage cancer cell lines protected against oxidative agents-induced cytotoxicity.

Reduction of cell viability can be caused by various mechanisms including apoptosis. Previous study reported that curcumin inhibited H_2O_2-induced apoptosis in Neuro-2A cells by reducing intracellular ROS levels,[11] a finding which we also observed in our study. Thus, we hypothesized that curcumin prevents H_2O_2-induced cytotoxicity in part via a reduction in apoptosis. Indeed, we found that curcumin at the concentrations of 5, 10, 15 and 20 μg/mL reduced DNA fragmentation, a characteristic of apoptosis. Expression of the pro-apoptotic protein BAX, which was induced by H_2O_2 treatment, was significantly decreased when the cells were pre-treated with 5, 10 and 15 μg/mL curcumin. Furthermore, pre-treatment by 15 μg/mL curcumin resulted in a reduction in both the levels of activated caspase-3 and its activity, confirming the anti-apoptotic effect of curcumin. Although caspase-3 activity in cells pre-treated with 5 and 10 μg/mL curcumin was not reduced significantly, viability of cells pre-treated with curcumin at those concentrations was significantly higher than that of H_2O_2-treated cells. This indicates that curcumin could also potentially inhibit a reduction in cell viability induced by H_2O_2 via another mechanism in addition to its anti-apoptotic activity.

It is known that curcumin, at a high concentration, is toxic to the cells.[16-18] In the PC12 cell culture model, curcumin concentration of at least 50 μg/mL was able to reduce cell viability by half.[10,18] In the present study, we found that addition of 100 μg/mL curcumin dramatically decreased cell viability to 38.88±10.98% compared to untreated control. Several pieces of evidence stated that the cytotoxicity of curcumin at high concentration is mediated via various mechanisms including cell proliferation inhibition and apoptosis induction.[19] For instance, curcumin, at high concentration, exhibited negative effect on cell proliferation in a glioma cell line U251.[20] High dose curcumin, especially in a presence of transition metal ions such as copper,[21-22] triggered oxidative stress and DNA damage, conditions that caused apoptosis, in human hepatoma HepG2 cells.[16] Thus, curcumin exerts either anti-apoptotic or pro-apoptotic effects, depending on its concentrations. However, *In vivo* toxicities of curcumin, would be unlikely since its toxicity levels would never be reached via oral administration.[9]

CONCLUSION

To summarize, our study showed that curcumin at an optimal concentration (15-20 µg/mL) possessed potent anti-oxidative and anti-apoptotic properties. H_2O_2-induced cytotoxicity in SK-N-SH cell line can be reversed by pre-treating cells with curcumin. Neuroprotective effect of curcumin is mediated in part via an inhibition of intracellular ROS accumulation, which consequently, leads to a reduction in H_2O_2-induced apoptosis as evident by a decrease in levels of pro-apoptotic protein BAX and activated caspase-3 as well as reduced caspase-3 activity. We, therefore, propose that curcumin has great potential for ameliorating neurocytotoxicity induced by H_2O_2. However, further studies such as *in vitro* study in primary rodent brain slices and human induced pluripotent stem cell-derived neurons are needed for validating the findings in neuroblastoma cell line. Additional *in vivo* preclinical research, e.g. study in Alzheimer's disease mouse model, would also be required for addressing pharmacokinetic properties, cytotoxicity, protective effect and, more importantly, neurobehavioral benefits of curcumin in a more relevant context.

ACKNOWLEDGMENTS

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Low Complication Rate Associated with Total Laparoscopic Hysterectomies Using the Retroperitoneal Approach: A Series of 1,092 Cases in Siriraj Hospital

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ABSTRACT

Objective: This study was performed to evaluate the incidence of and risk factors for major complications associated with the Siriraj total laparoscopic hysterectomy (SiTLH) technique.

Methods: In total, 1,092 patients who underwent SiTLH from January 2009 to December 2013 were enrolled in this retrospective study. The incidence of major complications such as death, vascular injury, visceral injury was the main outcome. Comparison between the patients with and without complications was such as unintended laparoconversion, vascular injury, visceral injury was the main outcome. Comparison between the patients with and without complications was performed to determine associated risk factors. Patient satisfaction was also evaluated.

Results: The incidence of major complications was 2.1%. There was a significantly increased risk of major complications among the patients with a uterine weight of ≥500 g (7.5% vs 1.8%, P =0.002), the patients with a uterine weight of ≥400 g and pelvic endometriosis (14.3% vs 1.9%, P =0.033), or experience of surgeon <15 years (2.9% vs 1.2%, P=0.049). In total, 1,031 (94.4%) of the patients were extremely satisfied with the results of surgery.

Conclusion: SiTLH technique is feasible and safe. However, the authors believe that good surgical skills and an understanding of the pelvic anatomy are essential to ensure good outcomes using our technique.

Keywords: Total laparoscopic hysterectomy; complication; associated factor (Siriraj Med J 2018;70: 191-197)

INTRODUCTION

Hysterectomy is the most common major gynecological procedure in Thailand. Traditionally, it is performed by laparotomy or a vaginal approach. However, during the past two decades, laparoscopic surgery has played an important role in hysterectomy since its initial report in 1989.[1] The advantages of laparoscopy include decreased postoperative pain, a more rapid return to normal activities, fewer wound complications, and a lower incidence of postoperative adhesion.[2] Johnson et al.,[3] compared the methods of hysterectomy and found a significantly quicker return to normal activities in association with vaginal hysterectomy. When vaginal hysterectomy is not possible, laparoscopic hysterectomy is preferable to abdominal hysterectomy, although this is at the cost of a greater chance of bladder or ureter injury. Conversely, several studies[4,5] found no significant differences in major complications between total laparoscopic hysterectomy (TLH) and total abdominal hysterectomy. This discrepancy is probably a result of the technical difficulty of the procedures, the steep learning curve, and selection bias.

We performed the first TLH in Siriraj Hospital in

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2004 and subsequently initiated a Siriraj TLH technique (SiTLH) in 2006. The aims of the present study were to determine the complication rate of this novel technique and to evaluate the risk factors associated with these major complications.

MATERIALS AND METHODS

This was a retrospective, descriptive study. Medical records were retrieved from the Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University.This study was reviewed and approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University before the study began (Si 004/2015). All patients who underwent SiTLH by the staff working in the Thai-German Multidisciplinary Endoscopic Training Center between 5th January 2009 and 27th December 2013 were eligible. Patients with incomplete medical records were excluded. Informed consent was not required because of the retrospective nature of the study.

In total, 1,092 patients who met our criteria were recruited for the study. Data collection included preoperative, intraoperative, and postoperative characteristics of the patients. The operative time was defined as the duration from skin incision to wound closure. The uterus was weighed immediately after the operation. Major complications were defined as death, vascular injury, visceral injury, unintended laparoconversion, massive blood loss requiring blood transfusion, and vaginal cuff bleeding or dehiscence requiring readmission or surgical intervention. Laparoconversion was described as the need to change from laparoscopic surgery to laparotomy. Blood loss was measured by the difference between the volume of fluid in the suction equipment and the volume of saline solution used during irrigation. Organ injuries were defined as those requiring surgical correction. Massive blood loss was defined as a total blood loss of >1,000 ml. Postoperative fever was defined as a temperature of at least 38°C, measured on two separate occasion at least 12 hours apart, after the first day of the postoperative period. Three months postoperatively, all patients were assessed regarding their long term complication and satisfaction level on a Likert-type scale[6] of 1 to 5 by a telephone interview and feedback.

The incidence of major complications was determined as the main outcome. Comparison of patients with and without complications was performed to identify associated risk factors. All statistical analyses were performed using SPSS version 13 software (SPSS Inc., Chicago, IL, USA). Demographic data are presented as number (percentage), mean ± standard deviation, and median (range). Student's t test, the Chi-square test, and Fisher's exact test were used to compare the two groups. A *P* value of <0.05 was considered statistically significant.

Siriraj TLH technique (SiTLH)

We developed a new surgical technique to reduce the rate of internal organ injury and blood loss during TLH. The principles of our technique include early identification of both ureters at the beginning of surgery, dissection of the ureter and uterine artery in the retroperitoneal space, ligation of the uterine artery to reduce the blood supply to the uterus, and restoration of the pelvic anatomy from the adhesion-free area to the adhesion area.

All laparoscopic procedures were performed under general anesthesia with the patient in the lithotomy position. A sound-tenaculum uterine manipulator was placed. Bladder drainage was established by insertion of a 14-French Foley catheter. The abdominal entry technique was introduced with the Veress needle/trocar technique. A10-mm laparoscopic trocar was inserted at intraumbilical area for the optic, and two or three 5-mm trocar at both iliac and suprapubic regions for ancillary instruments. In difficult cases, the10-step SiTLHwas routinely performed as follows. (I) The round ligament was grasped, bipolarly cauterized, and cut downward to the vesico-uterine peritoneum. (II) The bladder was dissected from the lower uterine segment. (III) The posterior leaf of the broad ligament was opened, and the infundibulopelvic ligament or the ovarian ligament was tied with 1-0 Polyglactin, bipolarly cauterized, and cut depending on whether the ovaries were being preserved. (IV) The retroperitoneal space was created. (V) The uterine artery was isolated and ligated at its origin with 1-0 Polyglactin. (VI) The ureter was identified, and ureterolysis was performed from the cephalic to caudal part as shown in Fig 1. The same procedure was also performed on the other side. (VII) In patients with severe pelvic adhesion such as those with severe pelvic endometriosis, restoration of the pelvic anatomy commenced from the adhesion-free area to the adhesion area. Dissection between the rectum and posterior wall of the uterus was achieved by creation of the medial pararectal space and rectovaginal space as shown in Fig 2. (VIII) Both cardinal ligaments were sutured above the ureter with 1-0 Polyglactin, then bipolarly cauterized and cut as shown in Fig 3. (IX) The vaginal tube was inserted. Colpotomy was performed with a monopolar hook at the level of the vaginal fornix. If the uterus was large, the specimen was morcellated via the 11-mm trocar with morcellator. The rest of the uterus was removed through the vagina. (X) The vaginal vault was sutured laparoscopically with 1-0 Polyglactin.

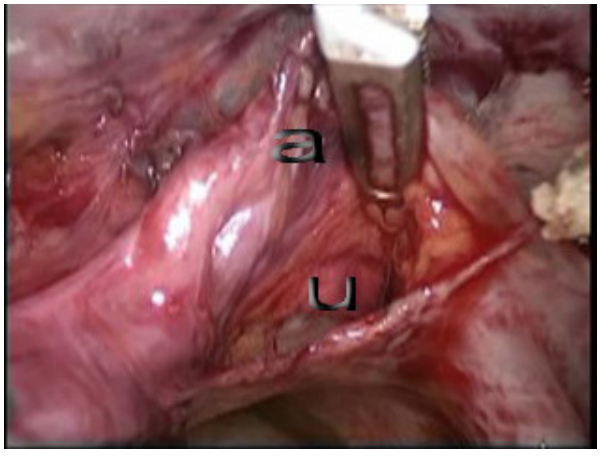


Fig 1. Retroperitoneal dissection showed uterine artery (a), ureter (u).

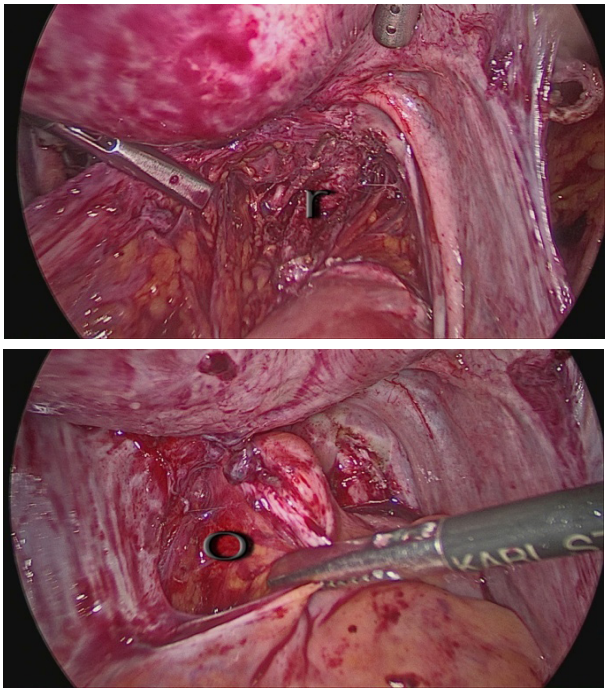


Fig 2. Dissection between the rectum and posterior wall of the uterus was archived by creation of the medial pararectal space (o) and rectovaginal space (r).

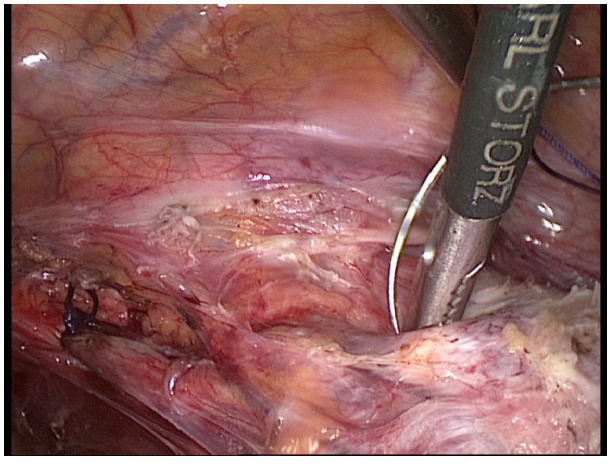


Fig 3. Cardinal ligament was sutured above the ureter with 1-0 Polyglactin.

RESULTS

In total, 1,092 patients who underwent SiTLH were enrolled during the study period. With the mean age of 43.6 years and the mean body mass index of 23.4 kg/m^2, more than one third of them had a history of previous surgery. The most common previous surgery was cesarean section (21%). Endometriosis was the most common main diagnosis (44.5%) followed by myoma uteri (38.5%) (Table 1).

With the median intra-operative blood loss of 50 ml and the mean length of hospital stay after surgery of 2.8 days, 23 of them (2.1%) had major complication (Table 2). Most of the major complications were detected intra-operatively, whereas four of them were detected after discharge.Nearly half of major complications (11/23) was bladder injury. A case of unintended laparoconversion due to uncontrollable massive bleeding was observed. The most common late complication was vaginal cuff dehiscence (Table 3).

Risk factors for major complications were the specimen weight of ≥500 g, the specimen weight of ≥400 g with pelvic endometriosis, and experience of surgeon <15 years (Table 4). By subgroup analysis, neither previous cesarean section, uterine weight ≥500 g nor previous cesarean section with uterine weight ≥500 g was correlated with bladder injury (p=0.708, 0.503, 1.00, respectively). Moreover, pelvic endometriosis was not associated with ureteric injuries (p=1.00). Not only did the experience of surgeon significantly reduce the major complications, but also massive blood loss as shown in Figs 4 and 5, respectively.

DISCUSSION

The major complication rate SiTLH in the present study was 2.1%. It represents an acceptable outcome, compared with many previous studies.[4,5,7,8] A systemic review[7] found the total complication rate of TLH varied from 0.6 to 18.0%. Additional to surgeon's experience and surgical technique, these variations depend on the inclusion/exclusion criteria, disease pathology, surgical instruments, and patient characteristics. Compared with conventional TLH technique, TLH with retroperitoneal approach had a low complication rate.[7-9] The complication rate and the number of patients in the present study were comparable with that of the report of Kobayashi et al.[8] They reported TLH with retroperitoneal approach in 1,253 patients using an early ureteral identification technique with 0.4% of ureteric injury, and 181 ml of mean blood loss. SiTLH was a retroperitoneal approach technique, not only invented to reduce ureteric injury by ureteral identification and/or ureterolysis, but also this

TABLE 1. Baseline characteristics (N= 1,092).

Characteristics	Values[a]
Age, y	43.6 ± 10.3
Body mass index[b]	23.4 ± 4.5
Morbid obesity	94 (8.6)
Previous surgery	390 (35.7)
Main diagnosis	
Severe endometriosis	486 (44.5)
Myoma uteri	420 (38.5)
Others	186 (17.0)
Additional surgical procedures	
Adnexal surgery	660 (60.5)
Lymph node dissection/sampling	20 (1.8)
Excision of deeply infiltrating endometriosis	14 (1.3)
Others	37 (3.4)

[a] Values are given as mean ± standard deviation or number (percentage).
[b] Calculated as weight in kilograms divided by the square of height in meters.

TABLE 2. Operative outcomes (N = 1,092).

Outcomes	Values[a]
Readmission	5 (0.5)
Reoperation	4 (0.4)
Length of hospital stay, d	2.8 ± 1.3
Operative time, min	150 (120, 180)
Specimen weight, g	200 (120, 300)
Blood loss, ml	50 (30, 100)
Blood transfusion	5 (0.5)
Total complication[b]	45 (4.1)
Major complication[b]	23 (2.1)
Minor complication[b]	22 (2.0)
Patient satisfaction	
Extremely satisfied	1,031 (94.4)
Very satisfied	35 (3.2)
Moderately satisfied	6 (0.5)
Slightly satisfied	12 (1.1)
Not all satisfied	8 (0.7)

[a] Values are given as mean ± standard deviation, number (percentage) or median (interquartile range).
[b] Calculated as number of patients who had a complication divided by total number of patients.

TABLE 3. Complication rates associated with laparoscopic hysterectomy.

Complication rate	Values[a]
Major complication[b]	23 (2.1)
Early complications	
Bladder injury[c]	11 (1.0)
Ureteric injury[c]	2 (0.2)
Bowel injury[c]	1 (0.1)
Massive blood loss with/without blood transfusion[c]	7 (0.6)
Unintended laparoconversion[c]	1 (0.1)
Late complications	
Vaginal cuff dehiscence[c]	3 (0.3)
Ureteric stenosis[c]	1 (0.1)
Minor complication rate[b]	22 (2.0)
Fever[c]	16 (1.5)
Shoulder/epigastric pain[c]	11 (1.0)
Nausea/vomiting[c]	5 (0.5)
Urinary retension[c]	1 (0.1)
Vaginal cuff infection[c]	1 (0.1)
Total complication[b]	45 (4.1)

[a] Values are given as number (percentage).
[b] Calculated as number of patients who had a complication divided by total number of patients.
[c] Calculated as number of events divided by total number of patients.

TABLE 4. Risk of the major complications associated with various risk factors.

Risk factors	Yes	No	*P* value
Specimen weight of ≥ 500 g	5/67 (7.5)	18/1,025 (1.8)	0.002
Presence of pelvic endometriosis	10/486 (2.1)	13/606 (2.1)	0.630
Specimen weight of ≥ 400 g with presence of pelvic endometriosis	2/14 (14.3)	21/1,078 (1.9)	0.033
Surgeon experience of < 15 years	17/586 (2.9)	6/506 (1.2)	0.049
Lymph node dissection/sampling	0/20 (0)	23/1,072 (2.1)	1.000
Morbid obesity	3/94 (3.2)	20/998 (2.0)	0.443
Previous abdominal surgery	10/390 (2.6)	13/702 (1.9)	0.432

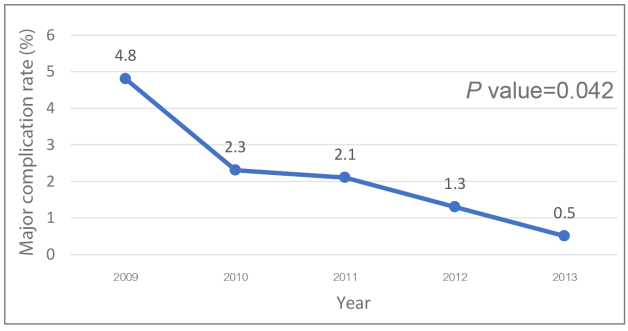


Fig 4. Major complication rate by the year of surgery.

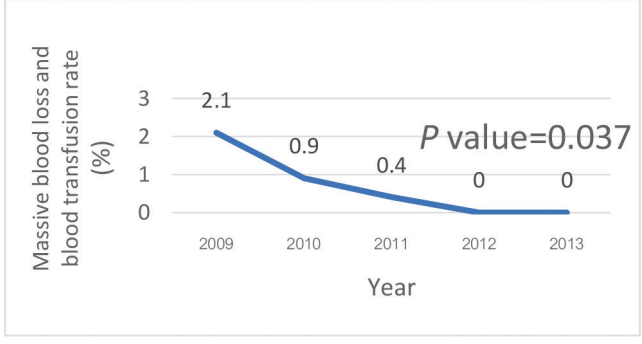


Fig 5. Massive blood loss with blood transfusion rate by the year of surgery.

technique intended to reduce blood loss by early ligation of major blood supply to uterus at the beginning of the procedure reducing the rate of ureteric injury, and the median blood loss, so the rate of blood transfusion in the present study was very low, 0.2%, 50 ml and 0.5%, respectively. Since, nearly half of the cases in the present study had severe pelvic endometriosis, they underwent difficult laparoscopic surgical procedure.This could be a benefit of SiTLH technique, because TLH in this pelvic inflammatory and adhesive disease resulted in increase in ureteric injury, intraoperative blood loss and blood transfusion.[10]

Many surgeons ignore to perform early ligation of both uterine arteries and infundibulopelvic (IP) ligaments. By the author's experience, doing early ligation can dramatically reduce blood loss from many raw surface areas fed by small branches of these major vasculatures. Moreover, the benefit of both IP ligament ligations before coagulation and cutting them in SiTLH technique can absolutely prevent the venous reflux. Without IP ligament ligation, the venous reflex from these ligaments is commonly found during the surgical process. It is not only unnecessary blood loss, but it also hinders the maintenance of the surgical field leading to increased risk of retroperitoneal structural injuries.

Basic laparoscopic surgery such as diagnostic laparoscopy is sufficiently mastered during residency training, although not for the more advanced procedures. Twenty cases were required to guarantee competency, whereas 30-50 cases were required to master a specific procedure such as TLH.[11] All surgeons in the present study had performed more than 30 cases of conventional TLH before SiTLH was invented. Major complications, massive blood loss and blood transfusion rate in the present study were significantly decreased by the years of surgery. It represented that SiTLH required deep and steep learning curve for getting familiar in this sophisticated technique to gain more experience.

In difficult cases, early identification of both ureters, retroperitoneal space dissection, routine ureterolysis and dissection from the adhesion-free area to the adhesion area in this technique can decrease the rate of ureteral injury. The rate of ureteric injury in the present study was only 0.2%, compared with a rate of 2.0% to 2.5% in previous studies.[4,12] However, the authors found a case of moderate hydronephrosis after 3 months of surgery. She underwent SiTLH technique with extensive right ureterolysis due to severe pelvic endometriosis with severe retroperitoneal adhesion. Further studies are needed to verify the long-term effect of ureterolysis in case of severe retroperitoneal adhesion.

Interestingly, bladder injury was the most common complication in the present study, comparable to the previous studies.[7,13] Lafay Pillet MC et al.,[14] demonstrated the rate of bladder injuries was 1%. The risk factors were previous caesarean section and previous laparotomy. However, the rate of injury reduced with the surgeons' experience and reached a plateau of 0.4% after 100 hysterectomies performed. Of 11 cases of bladder injury, 3 patients had a history of previous cesarean section, 2 patients had a uterine weight ≥ 500 g, 2 patients had bladder endometriosis, and 7 patients were operated on by surgeon that had an experience less than 15 years. In the present study, only surgeon experience was associated with bladder injury (p=0.17), whereas the others were not. However, our study had a small sample size to detect such a complication when subgroup analysis was applied.

A meta-analysis reported the low incidence of vaginal dehiscence was 0.64%. However, this extremely serious post-operative complication, with potential heavy morbidity of patients, needs urgent intervention otherwise the severe sequelae such as bowel gangrene, and pelvic peritonitis might be developed. Laparoscopic vaginal suturing was found to be the only risk factor of this complication, whereas use of monopolar or reducing the power energy did not alter the rate of cuff separation.[15] In the present study, there were only three vaginal cuff

dehiscences (0.3%) found. Interestingly, all of them had this kind of complication during sexual intercourse at 2 months after surgery. Hence, our patients were advised to restrain from sexual intercourse for at least 3 months postoperatively. Additionally, triple layer vaginal vault closure technique was developed in 2015 to reduce this severe post-operative complication to be zero. Further studies are needed to verify the long-term effect of this type of procedure.

Several studies[16-19] demonstrated that TLH is feasible and safe for selected patients with uteri weighing >500 g. On the contrary, specimen weight of ≥500 g was a risk factor associated with major complications in our study. This discrepancy may be related with more varieties of surgical experience and more coexisting pelvic endometriosis. Most of our complicated cases were performed by less experienced surgeons (74%) and up to 43% of cases had coexisting severe pelvic endometriosis. The large uterus coexisting with severe pelvic endometriosis affected the mobility of the uterus during surgery. Bladder injury was the most common complication in the present study. After analysis, we found that it associated with less experienced surgeons and incorrect anatomical dissection was the major cause.

Additional to the number of sample size, the variations in surgical skill, and long-term follow-up for at least 3 months to detect late complication were our strengths. Moreover, coexisting pathologies such as severe pelvic endometriosis and the oncologic operations such as pelvic and para-aortic lymphadenectomy were used for determination.

In conclusion, SiTLH technique in experienced hands can decrease the complication rate especially the massive blood loss volume or blood transfusion rate and reduces the ureteric injury.

Conflict of Interest: The authors declare no conflicts of interest.

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Transarterial Embolization: Alternative Treatment of Early and Late Postpartum Hemorrhage at a Single Tertiary Center

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ABSTRACT

Objective: To evaluate the efficacy of uterine artery embolization (UAE) in the treatment of postpartum hemorrhage (PPH).

Methods: All women who underwent UAE for the treatment of PPH between August 2008 and February 2016 were included. The patients were divided into early and late PPH defined by the onset of bleeding. All UAE procedures were performed by experienced interventional radiologists. Electronic medical records and radiological images were reviewed for data collection. Statistically significant associations between angiographic findings and the onset of PPH were evaluated.

Results: Total twenty-one women were enrolled in the study. The mean age of the patients was 30.1 years (range 16-42 years).Total 23 UAE procedures were performed in 21 women. Major clinical presentation was vaginal bleeding in 19 patients (90.5%) and hypovolemic shock was found in 10 patients (47.6%). Eight patients (38.1%) were in early PPH group and thirteen patients (61.9%) were in late PPH group. Five women underwent hysterectomy before UAE. Active contrast extravasation was a common finding associated with early PPH (50%) while pseudoaneurysms were commonly found in late PPH (53.8%) (p=0.016). The overall technical and clinical success rate were 100%. However, two women required repeated UAE after gelatin sponge embolization for active extravasation due to re-bleeding. No immediate complication of UAE was found in our study.

Conclusion: UAE is a safe and effective treatment for both early and late PPH. Various embolic materials can be used but permanent embolic material is preferable in patient with extravasation.

Keywords: Postpartum hemorrhage; embolization; pseudoaneurysm (Siriraj Med J 2018;70: 198-203)

INTRODUCTION

Postpartum hemorrhage (PPH) is an important obstetric complication that causes maternal morbidity and mortality. PPH is defined as bleeding from the genital tract exceeding 500 ml for vaginal delivery or greater than 1000 ml for cesarean section, which can occur within the first 24 hours following delivery (early PPH) or after 24 hours (late PPH).[1-3] The common causes of PPH include uterine atony, retained placenta, coagulopathy, genital tract injury, and endometritis.

The treatments of PPH comprise of supportive and definitive management which includes giving intravenous fluid and blood components, uterine compression, uterotonic agent administration, and finally, hysterectomy in patients not responding to conservative treatment.[4-7] Transarterial embolization for treatment of PPH began to play a role in the current scenario due to it being a less invasive procedure with high success rate and significantly low complication rate.[2,8-10]

The purpose of this study was to analyze and report the efficacy of uterine arterial embolization (UAE) in the treatment of PPH at a single tertiary-care hospital.

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MATERIALS AND METHODS

Patient Selection

This retrospective study was conducted with the approval of the institutional review board of our institution with waiver of patient's informed consent (Si 344/2016). All women who underwent UAE for the treatment of PPH from August 2008 until February 2016 were included in this study.

A total of 21 women underwent UAE for the treatment of PPH in our institution during the study period. The patient characteristics were shown in Table 1. The mean age of the patients was 30.1 years (range 16-42 years); twelve patients were primiparous, and nine patients were multiparous. Three patients had unassisted vaginal deliveries, one patient had instrumented vaginal delivery, and 17 patients underwent cesarean section.

UAE Techniques

All of these patients were referred to the interventional radiology unit for UAE after all of the usual obstetric treatments of PPH were exhausted without gaining expected outcome. The usual obstetric treatments in our center depend on the cause of PPH, and it includes intravenous uterotonic agent injection, aggressive uterine massage, manual extraction of the retained placenta, and repair of genital lacerations.

The UAE for the treatment of PPH was performed in an interventional radiology unit. UAE was routinely performed in our center through a 5-Fr vascular sheath (Terumo, Somerset, NJ) placed via the right common femoral artery access site. A 5-Fr diagnostic catheter (Cobra C2, Terumo, Somerset, NJ) with a 0.035 guidewire (Terumo, Somerset, NJ) were used to catheterize the internal iliac artery, and then, angiography was performed. Superselection into uterine arteries was done by using a 2.7-Fr Progreat microcatheter (Terumo, Somerset, NJ), and angiography was performed. After satisfactory microcatheter position was confirmed within the required branch of the uterine artery via angiography, transarterial embolization was performed. Various kinds of embolic materials were used according to vascular pathology, angiographic findings, position of microcatheter and patient condition. The endpoint of embolization was the absence of a contrast extravasation or pseudoaneurysm in case of the angiographic findings positive of those vascular pathology. In the case of hyperemia of uterus without vascular pathology, stasis of flow in the uterine artery was the endpoint in post embolization angiography. Embolization technique in case of prior hysterectomy and uterine ligation was same as those who had not undergone hysterectomy.

Data analysis and statistics

PPH was categorized into two groups according to the onset of hemorrhage, early PPH was defined as hemorrhage within the first 24 hours after delivery. Late PPH was defined as hemorrhage occurring after 24 hours of delivery.

TABLE 1. Patient demographics and characteristics

Characteristics	N (%)
Age, mean ± SD (range)	30.1 ± 7.6 (16-42)
Hematocrit level, mean ± SD (range)	27.1 ± 8.4 (9-38)
Parity	
Primiparous	12 (57.1)
Multiparous	9 (42.9)
Mode of delivery	
Cesarean section	17 (81.0)
Vaginal delivery	4 (19.0)
Onset of PPH	
Early	8 (38.1)
Late	13 (61.9)
Clinical presentation	
Bleeding per vagina	19 (90.5)
Intraabdominal bleeding	2 (9.5)
Hypovolemic shock	10 (47.6)

Abbreviation: PPH = postpartum hemorrhage

For each patient, the hospital medical records and radiological images were reviewed. Demographic data collection included the indication for the procedure, patient's age, parity, obstetric history, mode of delivery, the timing of PPH in relation to time of the delivery, pre-procedural hematocrit level, and type of transfusion requirement before UAE. Specific UAE procedural data collection included the type of embolic material used, pre- and post-embolization angiographic findings, and associated procedural complications.

Technical success was defined as successful catheterization of pathologic arteries with embolization to stasis of a specific vascular lesion (i.e., extravasation, pseudoaneurysm or hyperemia).

Clinical success was defined as an improvement of the patient's condition and obviation of repeated UAE or surgical management post-UAE.

Using Fisher exact tests, statistically significant associations between subject characteristics (early vs late PPH) and angiographic findings were evaluated. Statistical analysis was performed with SPSS statistical package version 18.0. A *p*-value of lower than 0.05 was taken as statistically significant finding.

RESULTS

In total 23 UAE procedures were performed in 21 women. Major clinical presentation was vaginal bleeding in 19 patients (90.5%) and hypovolemic shock was found in 10 patients (47.6%). Eight patients (38.1%) underwent UAE for early PPH. Among these patients, uterine atony was diagnosed in 5 patients (62.5%). Four patients experienced disseminated intravascular coagulation, and one patient was affected by HELLP syndrome. Five patients in this group underwent subtotal hysterectomy before UAE as the first line management of PPH from uterine atony and placenta accreta.

The mean hematocrit level measured immediately before embolization was 27.1±8.4% (range 9-38%) regardless of whether they had been transfused or not. Sixteen women were transfused with packed red blood cells, ten with fresh frozen plasma, and six with platelet transfusions prior to the procedure.

Association between angiographic findings and onset of PPH was shown in Table 2. Four patients (50%) with early PPH had active contrast extravasation on pre-embolization angiography. Hyperemia and pseudoaneurysm were found in three (37.5%) and one (12.5%) patients, respectively (Figs 1, 2).

TABLE 2. Association between angiographic findings and onset of PPH

Onset of PPH	Angiographic findings, N (%)			
	Extravasation	Pseudoaneurysm only	Hyperemia only	P-value
Early	4 (50.0)	1 (12.5)	3 (37.5)	0.016
Late	0 (0.0)	7 (53.8)	6 (46.2)	

Abbreviation: PPH = postpartum hemorrhage

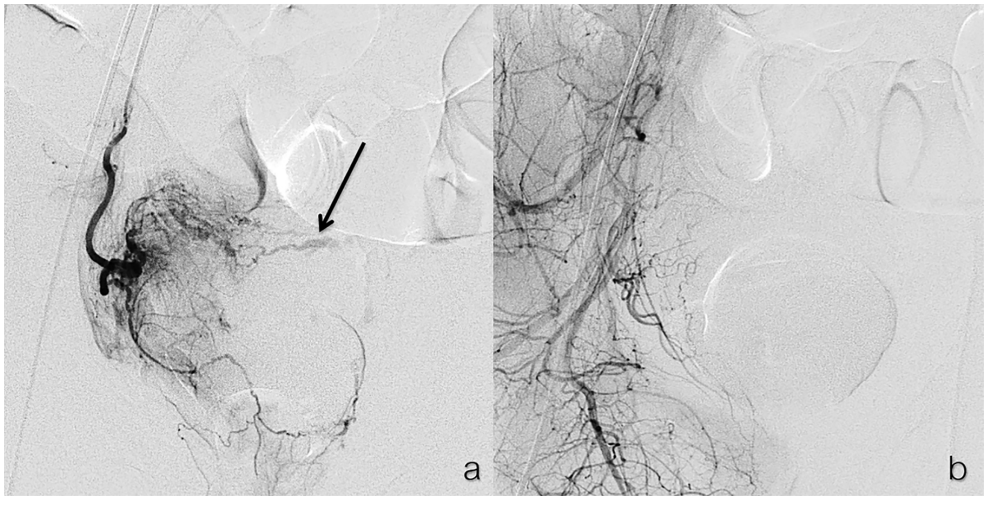


Fig 1. A 28-year-old woman with early PPH after cesarean section. (a) Selective angiography of the right uterine artery demonstrates active contrast extravasation (arrow). (b) Post-embolization angiogram after selective PVA embolization of the right uterine artery shows stasis of blood flow and no visualization of the active contrast extravasation.

Abbreviations: PPH = postpartum hemorrhage, PVA = polyvinyl alcohol

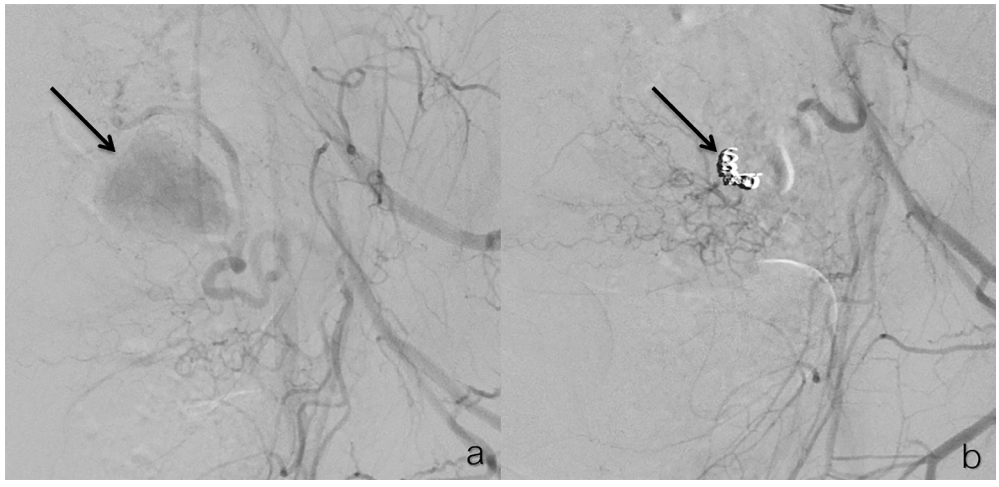


Fig 2. A 24-year-old woman with late PPH after cesarean section. (a) Selective pre-embolization angiography of the left uterine artery demonstrates a large pseudoaneurysm (arrow). (b) Post embolization angiogram after selective coil embolization (arrow) of the left uterine artery shows the non-visualization of the pseudoaneurysm.

Abbreviation: PPH = postpartum hemorrhage

Technical success and clinical success rate of UAE in PPH were 100% and 90.5%, respectively. There were two patients who needed a repeated embolization within 24 hours due to recurrent per vaginal bleeding after the first embolization. Both of them underwent hysterectomy before UAE. One of the patients had a vaginal delivery while another one had a cesarean section. Both patients had active contrast extravasation on the pre-embolization angiography of the first UAE session. They were embolized using gelatin sponge until disappearance of contrast extravasation was noted. However, the repeated angiography showed recurrent contrast extravasation at the previously embolized site in both patients. Coil and NBCA embolizations were done during the repeated embolizations (Fig 3). In the other two patients with active contrast extravasation, polyvinyl alcohol (PVA) and NBCA were used for embolization.

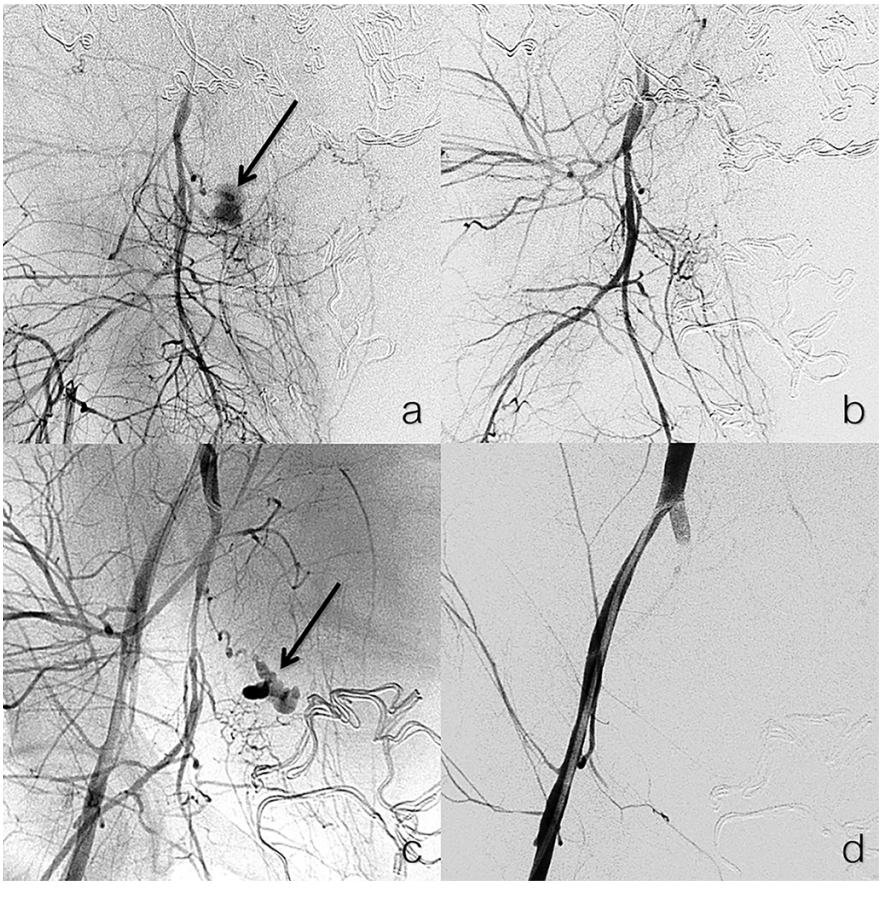


Fig 3. A 30-year-old patient with early PPH after normal vaginal delivery. (a) The initial pre-embolization angiography of right uterine artery demonstrates active contrast extravasation (arrow). (b) Post-embolization angiography after gelatin sponge embolization of the right uterine artery shows stasis of blood flow and no visualization of the active contrast extravasation. However, the patient developed recurrent per vaginal hemorrhage after embolization. (c) Selective right uterine angiography during the second UAE session shows recurrent active contrast extravasation (arrow) at the same location. The right uterine artery was re-embolized with NBCA. (d) Post-embolization angiography shows the disappearance of active contrast extravasation and lack of visualization of the terminal branches.

Abbreviations: PPH = postpartum hemorrhage, UAE = uterine artery embolization, NBCA = N-butyl cyanoacrylate

Thirteen patients (61.9%) underwent UAE for late PPH. One of the patients underwent uterine curettage before embolization for the removal of retained pieces of placenta. The most common angiographic finding in late PPH was pseudoaneurysm which was successfully embolized by the embolic material as shown in Table 3. No contrast extravasation was found in patients with late PPH at pre-embolization angiography. In all patients with angiographic findings of hyperemia only, non-selective embolization was done using gelatin sponge in the bilateral uterine arteries (Fig 4).

TABLE 3. Angiographic findings and types of embolic agent used

Angiographic findings	Embolic agents			
	Gelatin sponge	PVA	Coil	NBCA
Extravasation	2	1	1	2
Pseudoaneurysm	3	0	1	4
Hyperemia	9	0	0	0

Abbreviations: PVA = polyvinyl alcohol, NBCA = N-butyl cyanoacrylate

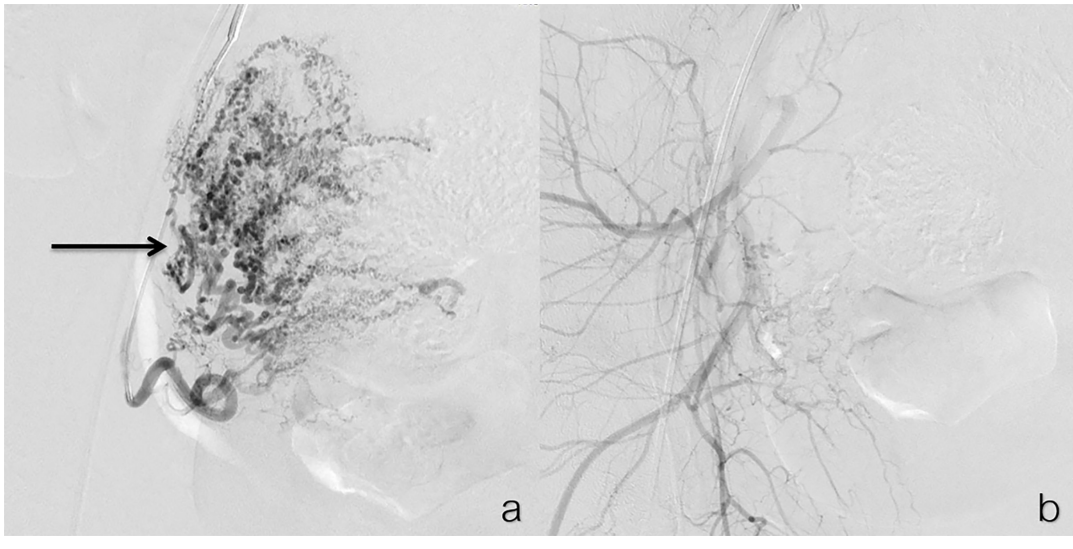


Fig 4. A 25-year-old woman developed endometritis with late PPH after cesarean section. (a) Selective pre-embolization right uterine angiography demonstrates hyperemia of the hypertrophied right uterine artery and its terminal branches (arrow) supplying the recently gravid uterus. No active contrast extravasation is identified. (b) Post embolization angiogram after selective gelatin sponge embolization of the right uterine artery shows stasis of blood flow and lack of visualization of the terminal branches.

Abbreviation: PPH = postpartum hemorrhage

DISCUSSION

PPH is a threatening cause of maternal morbidity and mortality. In most cases, obstetricians are able to prevent PPH with active management by using uterine massage and uterotonic therapy. When PPH occurs, multispecialty approaches are needed, including aggressive fluid resuscitation, massive blood transfusion, and application of uterine-sparing devices such as balloon tamponade, compression sutures, and arterial ligation.[5] If PPH cannot be controlled by these methods, hysterectomy remains as the definitive treatment for the patients.

However, the emergence of transarterial embolization as a treatment option in this group of patients helps in alleviating symptoms and reducing the number of hysterectomies. Brown et al[11] reported the first successful endovascular treatment for intractable PPH in 1979. Several more case reports followed with similarly encouraging results.[9] They helped to push endovascular treatment to the front line of management of PPH which failed conservative therapy.

In our study, the most common cause of early PPH requiring UAE is uterine atony (62.5%), which is same as reported in the previous studies.[12,13] Early PPH is a common presenting symptom of uterine atony. In most cases, uterine atony can be controlled with pharmacological interventions without the need for surgical interventions.

For late PPH, the common vascular pathology for UAE is pseudoaneurysm (53.8%). Although the other causes of late PPH such as endometritis and retained pieces of the placenta are more common than pseudoaneurysm in general populations, these conditions can be treated and bleeding controlled with pharmacological treatments and obstetric interventions. The presence of active contrast extravasation was associated with early PPH: meanwhile, the presence of a uterine artery pseudoaneurysm was associated with late PPH (p-value of 0.016). According to the natural history, active extravasation often presents with acute onset of bleeding and pseudoaneurysm usually presents with intermittent bleeding episodes.

In our study, we found that UAE was an effective treatment for control of intractable bleeding in both early and late PPH. The clinical success rates in this study were 90.5%, corresponding to previously published data which is between 73% and 100%.[12, 13] In clinical failure cases, two patients had a repeated UAE due to rebleeding in the case of extravasation, which were successfully treated by repeated embolization using permanent embolic materials. Park et al[14] reported the clinical failure after gelatin sponge embolization in a case of active extravasation. The literature review also showed that patients with active extravasation visualized angiographically were more likely to require repeated embolization ($P < 0.01$).[15] It is because gelatin sponge is a nonpermanent material which can spontaneously resolve over time leading to recanalization of the injured vessel. Therefore, gelatin sponge should not be recommended in cases of active extravasation or patients with coagulopathy.

According to the previously published data, the commonly encountered immediate UAE complications include arterial dissection, allergic reaction to the contrast medium used, hematoma at the puncture site, and pain.[10,16] Fortunately, there was no immediate complication in our study.

This study does have some limitations. Firstly, the study population was small, resulting in some findings which are statistically not significant. Secondly, we did not collect the long-term data of the patients including long-term complications and effects on fertility. We would suggest future studies in this field to include bigger sample populations and include a long-term follow up of these patients.

In conclusion, our results show that UAE should be the preferred treatment for intractable PPH when other conservative treatments have failed.

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Serum Neuropeptide Y and Leptin Levels compared between Non-pregnant and Pregnant Women in Overall, Non-obese, and Obese Subjects

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ABSTRACT

Objective: The primary objective of this study was to compare serum NPY and leptin levels between non-pregnant and pregnant women in overall, non-obese, and obese subjects. The secondary objective was to compare these peptides between non-obese and obese pregnant women.

Methods: Fasting venous blood was collected from non-pregnant women before open abdominal surgery and from pregnant women when admitted to the delivery room during the latent phase of labor.

Results: There were 12 non-obese and 14 obese subjects in the non-pregnant group and 9 non-obese and 30 obese subjects in the pregnant group. Systolic blood pressure (SBP) was comparable, but heart rate (HR) was higher in pregnant compared to non-pregnant women. Mean±S.E.M serum NPY levels were lower in the pregnant than in the non-pregnant group in overall (0.54±0.02 and 1.34±0.08, respectively), non-obese (0.53±0.05 and 1.23±0.14, respectively), and obese (0.54±0.03 and 1.43±0.09, respectively) subjects ($p<0.01$ for all), but these were comparable between obese and non-obese pregnant subjects. Serum NPY was positively correlated with SBP ($R=0.281$, $p<0.05$), but negatively correlated with HR ($R=-0.324$, $p<0.01$). Serum leptin levels were not different between pregnant and non-pregnant groups, but were significantly higher in obese than non-obese pregnant subjects ($p<0.001$). Serum leptin levels were positively correlated with body weight, BMI, waist and hip circumferences in overall and pregnant subjects ($p<0.001$ all).

Conclusion: In pregnancy, decreased NPY levels might be associated with inhibition of SBP rising as well as increased HR. Leptin levels might not be associated with pregnancy, but associated mainly with obesity.

Keywords: Neuropeptide Y; Leptin; non-pregnant; pregnant women (Siriraj Med J 2018;70: 204-212)

INTRODUCTION

Pregnant state is related with an increase in gestational weight gain (GWG) which is a unique and complex biological phenomenon that supports the functions of growth and development of the fetus.[1] The mean total GWG of normal weight adult women giving birth to term infants, is from maternal, placental, and fetal components, ranging from a low of 10.0 to a high of 16.7 kg.[1] For maternal component, fat appears to be increased in pregnant women, which preferentially deposits over the hips, back, and upper thighs throughout pregnancy.[2] Increased fat deposition leads to increased gene expression and secretion of certain adipokines from adipose tissue which are leptin and neuropeptide Y (NPY).[3,4]

Leptin is a peptide mainly produced and secreted from adipose tissue into the circulation in proportion to fat

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storage.[5-7] Leptin concentrations are positively correlated with indices of obesity including body weight, BMI, and waist and hip circumferences and is markedly increased in obese humans and in various animal models of obesity compared to controls or non-obese population.[8-11] Leptin acts as an afferent satiety signal to inhibit food intake[12] and stimulates energy expenditure.[13] In addition, leptin was found to be involved in blood pressure regulation. It increases sympathetic activity as chronic leptin infusion causes an increase in mean arterial pressure (MAP) and heart rate (HR) in mice.[14,15] On the other hand, acute and chronic leptin administration to rat aortic ring was shown to increase nitric oxide (NO) production and secretion leading to vasodilation.[16,17] In a pathological condition of obesity, chronic elevated serum leptin levels (hyperleptinaemia)[18] caused endothelial dysfunction and impaired NO-mediated vasodilatory effect of leptin[19] which led to increased blood pressure. The human placenta has been shown to be a source of leptin synthesis and secretion during pregnancy.[20] A previous study reported that circulating leptin levels and body fat were found to be higher during the early and late trimesters of pregnancy, than the pre-pregnant period[20] suggesting that the increase of this hormone might be from both placental and adipose production.

Neuropeptide Y (NPY), a 36 amino acid peptide[21], is the most potent orexigenic factor in the hypothalamus which increases body weight and adiposity by increasing food intake.[22] NPY levels in the circulation are also found to be higher in obese than non-obese or normal weight subjects.[4] NPY has another function implicated in cardiovascular regulation by modulating the sympathetic nervous system including vasoconstriction and control of heart rate and coronary blood flow.[23] Elevated NPY concentrations were also reported in hypertension.[24] NPY induces vasoconstriction directly by activation of its Y1 receptor, as well as indirectly by potentiating the effects of ATP and norepinephrine (NE) on adrenergic receptors.[25] Furthermore, plasma NPY concentrations were significantly correlated with BMI, waist circumference, HR, systolic blood pressure (SBP), and diastolic blood pressure (DBP).[26]

In summary, NPY and leptin are implicated in the regulation of food intake, body weight control, and cardiovascular modulation. The primary objective of this study was to compare serum NPY and leptin levels between pregnant and non-pregnant women categorized into non-obese and obese subgroups. As pregnancy is accompanied both by changes of adiposity and cardiovascular events, comparisons of their levels within the same BMI group might help to avoid the confounding effect of obesity. Furthermore, the secondary objective of this study was to compare serum NPY and leptin levels between non-obese and obese pregnant women to investigate the effect of obesity in pregnancy. Comparisons of these hormones between non-pregnant and pregnant women classified by BMI could reveal the changes of these peptides without the confounding effect of obesity. The changes of these peptides in pregnancy could probably explain physiological changes during pregnancy.

MATERIALS AND METHODS

Subjects

The study protocols were approved by the Siriraj Institutional Review Broad (Si 533/2009 for non-pregnant and Si 545/2015 for pregnant subjects) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Informed consents were obtained from all participants prior to the study. Sample size calculation for the comparison between the mean of 2 independent groups was obtained as follows: $n = 2 (Z_{\alpha/2} + Z_{\beta})^2/((\mu_1-\mu_2)/\sigma)2$, where $\alpha = 0.05$, 2 sided test, $Z_{\alpha/2} = 1.96$, $\beta = 0.2$, $Z_{\beta} = 0.842$, σ = common SD, $\mu_1-\mu_2$ = difference in mean, n = sample size per group. Sample size was obtained from previously published papers as 12 per group for NPY[27] and 2 per group for leptin.[28] In this study, there were 65 subjects who were classified into the pregnant group (n = 39) and the non-pregnant group (n = 26). For the non-pregnant group, recruited subjects were female patients undergoing open abdominal surgery. Phase of menstrual cycle of non-pregnant women could not be controlled because 23 subjects had myoma uteri and 3 subjects had adenomyosis which presented with irregular menstrual cycle. Exclusion criteria included subjects who underwent endocrine therapy (e.g., steroids, hormone replacement therapy, and thyroxine therapy) and had pregnancy, lactation, traumatic operation, malignancy diseases, operations related to endocrine diseases, severe abdominal inflammation, menopause, and/or hypertension. For the pregnant group, subjects who underwent either cesarean or normal labor, had antenatal care, and were in labor at Faculty of Medicine Siriraj Hospital, were healthy, and had age of at least 18 years old, gestational age of at least 34 weeks, and singleton, were recruited into this study. Exclusion criteria included subjects with gestational hypertension, human immunodeficiency virus (HIV) infection, type 1 or type 2 diabetes mellitus, metabolic syndrome, hypertension, polycystic ovarian syndrome, other endocrine disorders, previous history of chronic diseases, smoking habits, malignancies, pre-term membranes rupture, drug use that might affect blood glucose and insulin levels, and drug administration for

pre-term delivery risk and had fetuses with malformations and fetal distress during delivery.

Demographic data of subjects

Age of subjects and body mass index (BMI) of the non-pregnant group (range and median) were obtained from questionnaires and medical records. BMI of the pregnant women was calculated from pre-delivery body weight, which was obtained from medical records when subjects were admitted to the delivery room, subtracted by baby weight, placental weight, and estimated amniotic fluid weight. The estimated amniotic fluid weight was calculated as 6% of the gestational weight gain obtained from medical records as a previous study reported that amniotic fluid accounted for 6% of the gestational weight gain.[29] BMI of both non-pregnant and pregnant women were classified into non-obese (BMI < 25) and obese groups (BMI ≥ 25) according to the classification of BMI for Asian populations.[30] Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were measured by an automated sphygmomanometer. There were 12 non-obese and 14 obese subjects in the non-pregnant group and 9 non-obese and 30 obese subjects in the pregnant group.

Blood collection

Fasting venous blood samples were collected in the clot activator, silicone coated-tubes (BD Vacutainer®, Becton, Dickinson and Company Franklin Lakes, NJ, USA) during the fasting state from non-pregnant women before open abdominal surgery and from pregnant women when admitted to the delivery room during the latent phase of labor. For all subjects, venous blood had been drawn before intravenous fluid was given. Blood samples were immediately kept in an ice bucket during the transferring process and incubated at 4°C for 1 hour, balanced weight, and separated by centrifuging at 3,000 rpm at 4°C for 15 minutes. Then, serum samples were aliquoted and stored at -70°C, until analysis of serum NPY and leptin.

Serum NPY and leptin measurement

NPY and leptin serum levels were measured by commercial enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) kits (Phoenix Pharmaceuticals, Burlingame, California, USA), respectively. The protocol was done according to the manufacturer's procedures. The range of NPY detection was 0-100 ng/ml and the minimum detectable concentration was 0.11 ng/ml, with 100% and 14.3% cross-reactivity with neuropeptide Y (human, rat, mouse, porcine) and neuropeptide Y (3-36) (human, rat, mouse), respectively. Leptin detection range was 0.312-20.0 ng/ml, with 100% cross-reactivity with human leptin. For NPY, intra-assay variation was 8.84% for pregnant subjects and 10.1% for non-pregnant subjects, and inter-assay variation was 1.62%. For leptin intra-assay variation was 5.73% for pregnant subjects and 8.95% for non-pregnant subjects and inter-assay variation was 1.39%. Samples were run in duplicate and absorbance measured at 450 nm by Synergy HT Multi-Detection Microplate Readers (BioTek Instruments, Inc., Winooski, VT, U.S.).

Statistical analysis

Kolmogorov-Smirnov test was performed to test normality of the data. For normally distributed data, comparisons between the non-pregnant and pregnant groups as well as the non-obese and obese pregnant groups were performed by unpaired T-Test. Comparisons of non-normal distributed data were performed with a non-parametric test. Correlation coefficient was calculated by using the 2-tails Pearson product-moment correlation for normally-distributed data or the Spearman's rank correlation coefficient test for non-normally distributed data. Data were presented as mean±S.E.M. Statistical analysis was performed with PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA). A p-value less than 0.05 was considered as statistical significance.

RESULTS

Demographic and clinical data

Demographic data of the subjects including age and BMI are shown in range and median in Tables 1-4. Clinical data including SBP, DBP, MAP, and HR compared between the non-pregnant and pregnant groups in overall, non-obese, and obese subjects are shown as mean±S.E.M. in Tables 1, 2, and 3, respectively, as well as between non-obese and obese pregnant subjects in Table 4.

DBP was significantly higher in pregnant than non-pregnant women in overall subjects ($p<0.05$) (Table 1) and had a trend to be higher in pregnant than non-pregnant women in non-obese (Table 2) and obese groups (Table 3). HR was significantly higher in pregnant than non-pregnant women in the overall (Table 1), non-obese (Table 2), and obese groups (Table 3) ($p<0.05$ all). SBP and MAP were significantly higher in obese than non-obese pregnant women ($p<0.05$) (Table 4).

Serum levels of NPY and leptin compared between non-pregnant and pregnant women

Serum levels of NPY and leptin compared between

non-pregnant and pregnant women in overall, non-obese, and obese subjects are shown in Fig 1.

Serum NPY levels were significantly lower in the pregnant group than in the non-pregnant group in overall ($p<0.001$), non-obese ($p<0.01$), and obese ($p<0.0001$) subjects (Fig 1). Serum leptin levels were not different between the pregnant and non-pregnant groups in overall, non-obese, and obese subjects (Fig 1).

Serum levels of NPY and leptin in pregnant women compared between non-obese and obese subjects

Serum levels of NPY and leptin in pregnant women compared between non-obese and obese subjects are shown in Fig 2.

Serum NPY levels were comparable between non-obese and obese pregnant women (Fig 2). Serum leptin levels were significantly higher ($p<0.001$) in obese pregnant women compared to non-obese pregnant women (Fig 2).

TABLE 1. Clinical parameters of overall subjects compared between non-pregnant and pregnant women: number (n) of subjects (%), age and BMI (range and median), SBP, DBP, MAP, and HR (mean±S.E.M). *p<0.05, ***p<0.001

Parameters	Overall subjects (n = 65)		P-value
	Non-pregnancy	Pregnancy	
n (percentage)	26 (40%)	39 (60%)	-
Age (range), year	28-53	20-41	-
Age (median), year	45.0	28.0	-
BMI (range), kg/m^2	17.5-44.0	18.8-38.8	-
BMI (median), kg/m^2	26.3	27.5	-
SBP (mmHg)	118.1±2.0	118.2±1.0	0.956
DBP (mmHg)	72.8±1.7	77.1±0.8	0.026*
MAP (mmHg)	87.9±1.6	90.8±0.7	0.113
HR (bpm)	76.9±2.0	86.3±1.4	<0.0001***

TABLE 2. Clinical parameters of non-obese subjects compared between non-pregnant and pregnant women: number (n) of subjects (%), age and BMI (range and median), SBP, DBP, MAP, and HR (mean±S.E.M). *p<0.05

Parameters	Non-obese subjects (n = 21)		P-value
	Non-pregnancy	Pregnancy	
n (percentage)	12 (57.14%)	9 (42.86%)	-
Age (range), year	28-51	20-39	-
Age (median), year	43.5	27.0	-
BMI (range), kg/m^2	17.5-22.8	18.8-22.3	-
BMI (median), kg/m^2	20.6	21.7	-
SBP (mmHg)	117.3±3.9	114.4±2.4	0.812
DBP (mmHg)	70.6±2.2	74.4±1.8	0.239
MAP (mmHg)	85.0±1.8	87.8±1.7	0.935
HR (bpm)	81.1 ± 2.0	87.4 ± 1.8	0.034*

Correlations between 2 factors

Correlations between serum NPY and leptin with other factors in overall, non-pregnant, and pregnant subjects are shown in Table 5.

Serum NPY levels were significantly positively correlated with SBP (R=0.281, p<0.05), but significantly negatively correlated with HR (R=-0.324, p<0.01) in overall subjects. Serum leptin levels were significantly positively correlated to body weight, BMI, and waist and hip circumferences in overall and pregnant subjects (R=0.470-0.628, p<0.001 all). Serum leptin levels had a significant positive correlation with hip circumference in non-pregnant subjects (R=0.480, p<0.05).

TABLE 3. Clinical parameters of obese subjects compared between non-pregnant and pregnant women: number (n) of subjects (%), age and BMI (range and median), SBP, DBP, MAP, and HR (mean±S.E.M). ***p<0.001

Parameters	Obese subjects (n = 44)		P-value
	Non-pregnancy	Pregnancy	
n (percentage)	14 (31.82%)	30 (68.18%)	-
Age (range), year	28-51	21-41	-
Age (median), year	43.5	29.0	-
BMI (range), kg/m^2	25.2-44.0	25.1-38.8	-
BMI (median), kg/m^2	29.1	28.1	-
SBP (mmHg)	121.8±3.2	119.3±1.1	0.482
DBP (mmHg)	74.6±2.3	77.9±0.9	0.208
MAP (mmHg)	90.4±2.5	91.7±0.7	0.610
HR (bpm)	72.1±2.6	87.5±1.4	<0.0001***

TABLE 4. Clinical parameters of pregnant subjects compared between non-obese and obese pregnant women: number (n) of subjects (%), age and BMI (range and median), SBP, DBP, MAP, and HR (mean±S.E.M). *p<0.05

Parameters	Pregnancy (n = 39)		P-value
	Non-obese	Obese	
n (percentage)	9 (23.08%)	30 (76.92%)	-
Age (range), year	20-39	21-41	-
Age (median), year	27.0	29.0	-
BMI (range), kg/m^2	18.8-22.3	25.1-38.8	-
BMI (median), kg/m^2	21.7	28.1	-
SBP (mmHg)	114.4±2.4	119.3±1.1	0.044*
DBP (mmHg)	74.4±1.8	77.9±0.9	0.076
MAP (mmHg)	87.8±1.7	91.7±0.7	0.016*
HR (bpm)	87.4 ± 1.8	87.5±1.4	0.137

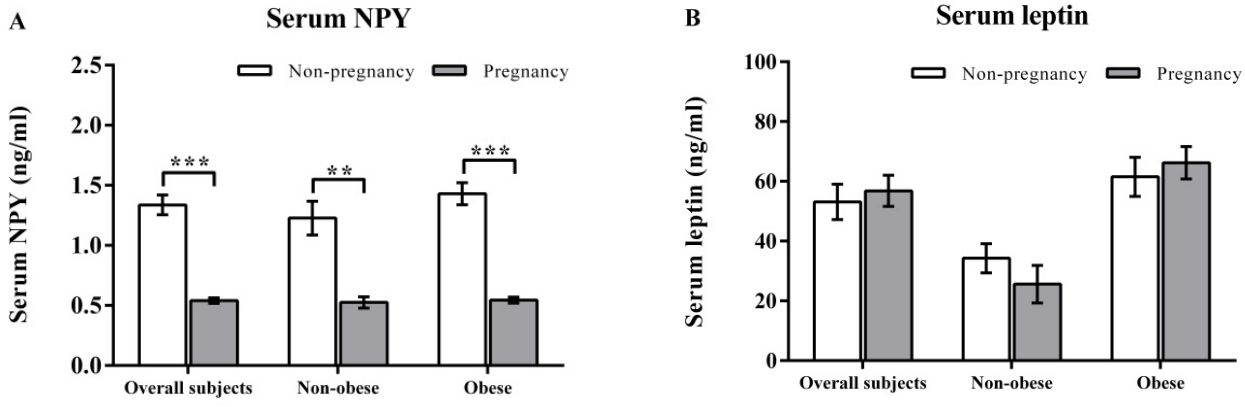


Fig 1. Serum levels of NPY and leptin compared between non-pregnant and pregnant women in overall, non-obese, and obese subjects. Panels A and B show serum NPY and leptin, respectively. Values are expressed as mean±S.E.M. **p<0.01, ***p<0.001 compared between non-pregnant and pregnant women.

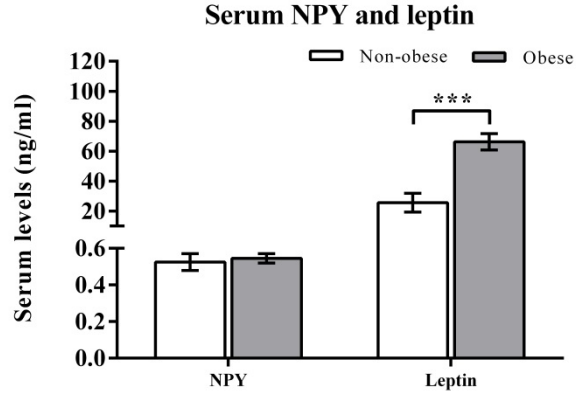


Fig 2. Serum NPY and leptin levels in non-obese and obese pregnant women. Values are expressed as mean±S.E.M. ***p<0.001 compared between non-obese pregnant and obese pregnant women.

TABLE 5. Correlations between serum NPY and leptin with other factors in overall, non-pregnant, and pregnant subjects.

R = correlation coefficient, SBP = systolic blood pressure, HR = diastolic blood pressure, BMI = body mass index, *p<0.05, **p<0.01, ***p<0.001

Factors	Overall subjects (n = 65)		Non-pregnant subjects (n = 26)		Pregnant subjects (n = 39)	
	R	P-value	R	P-value	R	P-value
Serum NPY						
- SBP	0.281	0.012*	0.035	0.850	0.131	0.379
- HR	-0.324	0.004**	-0.149	0.415	-0.066	0.665
Serum leptin						
- Body weight	0.545	<0.0001***	0.296	0.218	0.590	<0.0001***
- BMI	0.583	<0.0001***	0.366	0.124	0.645	<0.0001***
- Waist circumference	0.470	<0.0001***	0.211	0.386	0.618	<0.0001***
- Hip circumference	0.628	<0.0001***	0.480	0.038*	0.663	<0.0001***

DISCUSSION

This study focused on the comparisons of serum NPY and leptin levels between pregnant and non-pregnant women. During pregnancy, increased maternal adiposity might lead to changes of NPY and leptin levels, therefore, subgroup analysis by BMI classification was performed in this study to decrease confounding effects of obesity from pregnancy.

In this study, serum NPY levels were significantly lower in pregnant than non-pregnant women. DBP and HR were higher in pregnant than non-pregnant women while SBP and MAP were comparable among groups. Furthermore, NPY had a positive correlation with SBP but a negative correlation with HR. These results suggest that NPY levels in pregnancy were related to increased SBP, but decreased HR. A decrease in NPY levels in pregnancy might be associated with inhibition of SBP rising as well as increased HR. During pregnancy, there is a decrease in total peripheral vascular resistance[31,32] due to vasodilation of the renal and systemic circulation[33], although MAP is maintained[34] because there is a rise in blood volume and cardiac output by 30-50%.[31,32] As NPY has an effect to induce vasoconstriction, decreased NPY levels in pregnant women might lead to vasodilation in pregnancy to maintain uteroplacental circulatory blood flow in order to meet fetal metabolic demands.[35] The progressive increase in HR throughout pregnancy peaking in the late third trimester, likely counterbalances the vasodilation to maintain adequate cardiac output at a functional level until delivery. However, our results were inconsistent with previous studies showing higher NPY levels in pregnant compared to non-pregnant women. The inconsistency of the results might be due to BMI mismatch, different number of subjects, sampling population, and techniques used for analysis of NPY levels. For BMI mismatch, subjects of this study were classified into obese and non-obese as NPY levels are changed according to obesity status[4], while previous studies did not categorize subjects into obese and non-obese subgroups.[36,37] This might lead to unreliable results of the previous studies. Furthermore, to eliminate the confounding effect of obesity, the current body weight of pregnant subjects was calculated from subtraction of maternal body weight by baby weight, placental weight, and amniotic fluid weight. For number of subjects, our study had 26 non-pregnant and 39 pregnant subjects while previous studies had 15 non-pregnant and 33 pregnant subjects[36] and 8 non-pregnant and 8 pregnant subjects[37] which were lower than this study. For sampling population, non-pregnant subjects of our study were recruited from female patients undergoing open abdominal surgery. Twenty three subjects had myoma uteri and 3 subjects had adenomyosis presented with irregular menstrual cycle. On the other hand, other studies recruited healthy non-pregnant subjects without declaration of menstrual cycle. For the analytical methods of peptide concentrations, NPY levels were determined in serum by EIA technique in this study while in plasma by radioimmunoassay (RIA) in previous studies.[36,37] Furthermore, previous studies found that an increase in NPY levels from stress[38] as well as preeclampsia[37] was observed in platelet rich plasma (PRP), but not platelet poor plasma (PPP), suggestive of accumulation of the peptide in platelets.[39] As NPY levels were determined in serum in our study, NPY in platelets could not be measured probably leading to lower NPY levels than plasma.[39]

Serum NPY levels were comparable between non-obese and obese pregnant women and there was no correlation between NPY levels and obesity parameters including body weight, BMI, and waist and hip circumferences in this study. These results suggest that during pregnancy, NPY levels might be associated with hemodynamic regulation rather than obesity.

Serum leptin levels were not different between pregnant and non-pregnant women but were higher in obese compared to non-obese pregnant women. Furthermore, serum leptin levels had positive correlations with body weight, BMI, waist and hip circumferences. These results suggest that leptin levels were obviously associated with obesity rather than pregnant status. Our results were inconsistent with previous studies showing increased leptin levels in pregnant women than non-pregnant women.[20,40] In a previous study, leptin levels of pregnant women were reported without BMI matching at the time of blood sampling to non-pregnant women. As circulating leptin in pregnancy parallels the process of fat accumulation and mobilization[40], the increase in adiposity in pregnant women could lead to increased leptin levels in pregnancy. Our data supported the evidence showing that adipose tissue, not placenta, is a major source of circulating leptin production.[41]

In summary, this study revealed lower levels of serum neuropeptide Y in pregnancy compared to non-pregnancy in both non-obese and obese subjects. Thus, we postulated that, decreased neuropeptide Y levels during pregnancy might contribute to a decline in vasoconstriction leading to vasodilation. Serum leptin levels were higher in obese pregnant than non-obese pregnant women with comparable levels between BMI-matched non-pregnant and pregnant women. Therefore, serum leptin levels were associated with fat accumulation rather than a pregnant state.

One limitation of our study was that the exact body weight of pregnant women during the time of blood collection could not be obtained. However, the estimated body weight of pregnant group is a better representation of current BMI than pre-pregnant BMI or BMI at early gestational age. Estimated amniotic fluid weight used in this study was calculated from GWG instead of ultrasonography and operative record. This is because, in normal practice, the amniotic fluid measurement was reported as low, normal, and high amniotic fluid volume, not estimated into weight of amniotic fluid. As a result, we could not obtain amniotic fluid weight from this technique. Furthermore, this study recruited both normal and cesarean delivery, so the measurement of amniotic fluid during delivery was not applicable to all subjects. Therefore, estimated amniotic fluid weight from GWG might be the most convenient method in normal clinical practice setting. Another study limitation was that blood samples were collected before open abdominal surgery or before delivery which might lead to increased stress levels in the subjects. Another limitation of this study was that non-pregnant subjects were not healthy and had gynecological conditions including myoma uteri and adenomyosis.

The strengths of this study were that subgroup analysis of subjects into obese and non-obese was made and the current body weight of pregnant women was subtracted by baby weight, placental weight, and amniotic fluid weight to reduce the confounding effect of adiposity and GWG. The results of this study could be applied in a clinical context as an abnormal increase in NPY levels in pregnancy might lead to increased blood pressure resulting in pregnancy-induced hypertensive diseases including preeclampsia. Further studies will be needed to reveal the effect of decreased NPY induced vasodilation in normal pregnancy.

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Additional Hyaluronic Acid Binding Selection Decreased Sperm DNA Damage after Conventional Semen Preparation in Infertile Patient with Abnormal Semen Analysis

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ABSTRACT

Objective: To study the benefit of combining conventional sperm preparation methods with hyaluronic acid binding in selecting spermatozoa with less DNA damage in infertile patients with abnormal semen analysis.

Methods: Forty-six semen samples obtained from infertile men, containing two or more abnormalities regarding concentration, motility or morphology, were enrolled in the study. Baseline semen analysis was performed and recorded for all samples. Each sample was divided into 3 aliquots, i.e. the control group / fresh sample before preparation (Group 1), the conventional sperm preparation group including both density gradient centrifugation and swim-up (Group 2), and the conventional method combined with hyaluronic acid selection group (Group 3). The DNA integrity of sperm obtained by each different test group was assessed using TUNEL assay.

Results: Less sperm DNA damage was observed in Group 2 compared to Group 1, whereas Group 3 displayed significantly lower sperm DNA damage compared to Group 2 (9.4% vs. 27.0%, $p < 0.001$).

Conclusion: Sperm DNA damage was significantly reduced after the inclusion of hyaluronic acid binding in conventional sperm preparation method in abnormal semen samples.

Keywords: DNA damage; sperm preparation; density gradient centrifugation; swim-up; hyaluronic acid binding; TUNEL (Siriraj Med J 2018;70: 213-218)

INTRODUCTION

Infertility has developed into a public health issue affecting around 6.8-38.6% of reproductive couples worldwide, depending on different diagnostic criteria.[1] Male factor alone is estimated to be responsible for 20-30% of these cases.[2] Sperm quality is commonly described by the parameters of concentration, motility and morphology. Deviation of these parameters from the reference thresholds is correlated with poor quality and usually fertility problems.[3] Poor sperm quality (varying degrees of oligo-astheno-teratozoospermia) is frequently associated with increased sperm DNA fragmentation in infertile males.[4,5]

Many factors can influence semen quality, but the primary mechanism inducing DNA fragmentation is the post-testicular damage by free oxygen species, which usually occurs during sperm transportation through the epididymis.[6,7] However, normal semen samples obtained

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from infertile males, also display higher DNA fragmentation rate compared with fertile controls.[7,8] Sperm DNA damage may have several effects including fertilization failure and poor embryo development. Additionally, data supports the association of sperm DNA fragmentation with low live-birth rates,[9,10] increased pregnancy loss after IVF or intracytoplasmic sperm injection (ICSI),[11] poor embryo quality and lower implantation rate.[12-14]

ICSI is an ART tool designed to overcome fertilization failure of poor quality sperm. Sperm selection during ICSI usually depends on sperm motility and morphology. Nevertheless, motile spermatozoa with normal morphology may also carry fragmented DNA. This phenomenon is even more popular in poor quality semen samples.[4]

Several tests have been designed to assess DNA integrity, although none is generally accepted as the best diagnostic tool to predict or optimize clinical management.[15] Terminal deoxynucelotidyl transferase–dUTP nick end-labelling (TUNEL) assay is one of these methods providing great accuracy for quantifying apoptotic germ cells.[16,17] However, TUNEL is not suitable for DNA integrity assessment of viable sperm during ICSI, since the testing solutions used are toxic to the sperm.

Hyaluronic acid binding assay (HBA) is developed for selection of mature spermatozoa imitating the natural process of mature sperm binding on the cumulus oophorus complex of the oocyte which is composed of polymerized hyaluronic acid and glycoproteins.[18] Hyaluronic acid bound spermatozoa have been reported to carry low DNA fragmentation, less chromosomal aneuploidies and better nucleus morphology.[19,20] Hence, HBA method may be able to, indirectly, predict sperm DNA integrity while at the same time allowing the selected sperm to be used for ICSI thereafter. No adverse effects have been reported on either fertilization or embryo development following the use of hyaluronic acid bound spermatozoa in clinical IVF settings.[21]

The aim of this study was to compare the presence of DNA damage in spermatozoa retrieved by two different preparations methods prior to ICSI process, namely the conventional method (density gradient centrifugation followed by swim-up) and the conventional method combined with HBA method in infertile patients with abnormal semen analysis. The DNA fragmentation was evaluated by TUNEL assay.

MATERIALS AND METHODS

Study Design and Patient Selection

This was an experimental study, conducted at the infertility unit of a university hospital. The study protocol was approved by hospital institutional review board (Si 591/2012). The inclusion criteria contained healthy men, over 18 years of age, non- smokers, without medical history of receiving radiotherapy or chemotherapy and with abnormal semen analysis. Samples with two or more abnormal semen parameters (concentration, motility and morphology) according to World Health Organization (2010) criteria[3] were included. The subjects who had leukocytospermia or sperm motility < 5% were excluded from this study.

After baseline semen analysis was completed, each semen sample was divided into 3 aliquots. Group 1 used fresh smear as control. In Group 2, semen was prepared by the conventional method where density gradient centrifugation was followed by swim-up technique. In Group 3, semen prepared by the conventional method underwent HBA testing as well. Each group of semen sample was assessed for DNA damage by TUNEL assay.

Density gradient centrifugation and swim up

Density gradient conical tubes were prepared by adding 2 ml of 80% and 40% of percoll buffer (Percoll™ PLUS, GE Heathcare, Uppsala, Sweden) in lower and upper layers, respectively. The liquefied semen was layered onto the gradient. All samples were centrifuged at 300 x g for 20 minutes. Then the supernatant was gently removed, leaving the pellet at the bottom. A new pipette was used to aspirate the pellet and place it in 1ml of sperm media. For the swim up process 0.5 ml of Earle's balanced salt solution (Gibco™, Paisley, Scotland, UK) was placed over the sperm layer and was incubated in 37°C for 30 minutes. The sperm in the upper 0.5 ml was obtained for analysis or further treatment.

Hyaluronic acid binding method

The HA binding method was performed by placing 10 µL of SpermSlow™ media (Origio Medicult Media, Denmark) at the bottom of a pre-warmed at 37°C, ICSI dish. Then the prepared semen sample was placed close to the media. A junction between the sperm droplet and the SpermSlow™ was created by the tip of pipette. The ICSI dish was covered immediately with pre-equilibrated liquid paraffin oil and subsequently placed in a 5% CO_2, 37°C incubator for 15 minutes. The injection pipette was washed by pre-equilibrated sperm preparation medium before being used to select the spermatozoa. The motile sperm bound on HA were picked. Around 500 spermatozoa were collected and fixed for DNA assessment.

DNA damage analysis with the TUNEL assay

The DNA fragmentation was evaluated using the TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein,

Roche, Mannheim, Germany). The slides were initially fixed in 4% paraformaldehyde in Phosphate Buffer Saline solution for 1 hour. Then all slides were rinsed with phosphate buffer saline (PBS) and incubated in 0.1% triton- X 100 in 0.1% sodium citrate for 2 min on ice (2-8°C). Afterwards, slides were rinsed twice with PBS and incubated in 50 µL of TUNEL mixture in a humidified atmosphere, at 37°C, in the dark for 60 minutes before being washed three times with PBS. After being dried at room temperature, counterstain was performed with DAPI II solution (Abbott™, Ilinois, USA) which was then evaluated under fluorescence microscope (Imager-Z1; Zeiss, Göttingen, Germany). At least 500 spermatozoa were assessed per sample in random fields. TUNEL-positive spermatozoa exhibited a strong nuclear green fluorescence. DNA damage degree was expressed as a percentage of TUNEL-positive spermatozoa. The slides were assessed by two technicians and the mean of the results was used for calculation.

Statistical analysis

The degree of DNA damage between the two methods was assessed by Chi-square test. The correlation between semen parameters and DNA damage were analyzed by Pearson's correlation coefficient or Spearman's rank correlation in the data without normal distribution. The data analysis was performed using Statistical Programs for Social Sciences (SPSS Inc. version 15.0, Chicago, IL). A P value of < 0.05 was considered statistically significant. Sample size was calculated based on the data of a previous study,[20] which reported 11% DNA damage in swim-up technique and 5.3% in the HBA group. At type I error of 0.05 and allowable error of 0.1, at least 46 samples were needed for this study.

RESULTS

Totally 127 participants were eligible for the study. Eighty-one participants were excluded due to containing only one abnormal semen parameter. Therefore, forty-six participants were enrolled in the study. There were 34 semen samples with two abnormal semen parameters and 12 samples contained all three abnormalities on the aspect of concentration, motility and morphology. All subjects included in this study were recruited after providing informed consent.

The basic characteristics of all subjects and the semen parameters of the samples are summarized in Table 1. The mean age of participants was 34.9 ± 6.0 years. The mean abstinence time was 4.1± 2.2 days. The mean semen volume, concentration, motility and normal morphology was 2.1 ± 1.4 mL, 14.7 ± 18.8 x 10^6/mL, 26.0 ± 11.4% and 5.0 ± 3.6% respectively.

The DNA damage in fresh semen (Group 1) was measured at 39.0% by TUNEL assay. The sperm DNA damage in Group 2 (27%) was significantly lower than Group 1 ($P < 0.001$) and was markedly reduced in Group 3 (9.4%) compared to Group 2 ($P < 0.001$), as well. The fresh sperm DNA damage was similar in participants who had two (Group A) and three (Group B) abnormal semen parameters (38.9% and 39.3%, respectively). The conventional preparation significantly reduced DNA fragmentation in both Groups A and B to 27.1% and 26.8%, respectively. The addition of HBA reduced DNA damage in Group A and B even further to the lowest rate found in this study, i.e. 10.1% in Group 2 and 7.5% in Group 3 ($P < 0.001$) (Fig 1).

From univariate analysis for factors of sperm DNA damage, there was no linear relationship between semen parameters and DNA damage (Table 2).

TABLE 1. Basic characteristics of recruited subjects

	Mean ± SD	Median	Range
Age (years)	34.9 ± 6.0	35	24 – 50
Abstinent time (days)	4.1 ± 2.2	4	2 – 7
Semen volume (mL)	2.1 ± 1.4	1.60	1 – 7
Sperm concentration (million/mL)	14.6 ± 18.8	8	0.1 – 73
Sperm motility (%)	26.0 ± 11.4	27	3 – 50
Normal sperm morphology (%)	5.0 ± 3.6	4	0 – 16
WBC in semen (million/mL)	0.3 ± 0.2	0.2	0 - 0.8

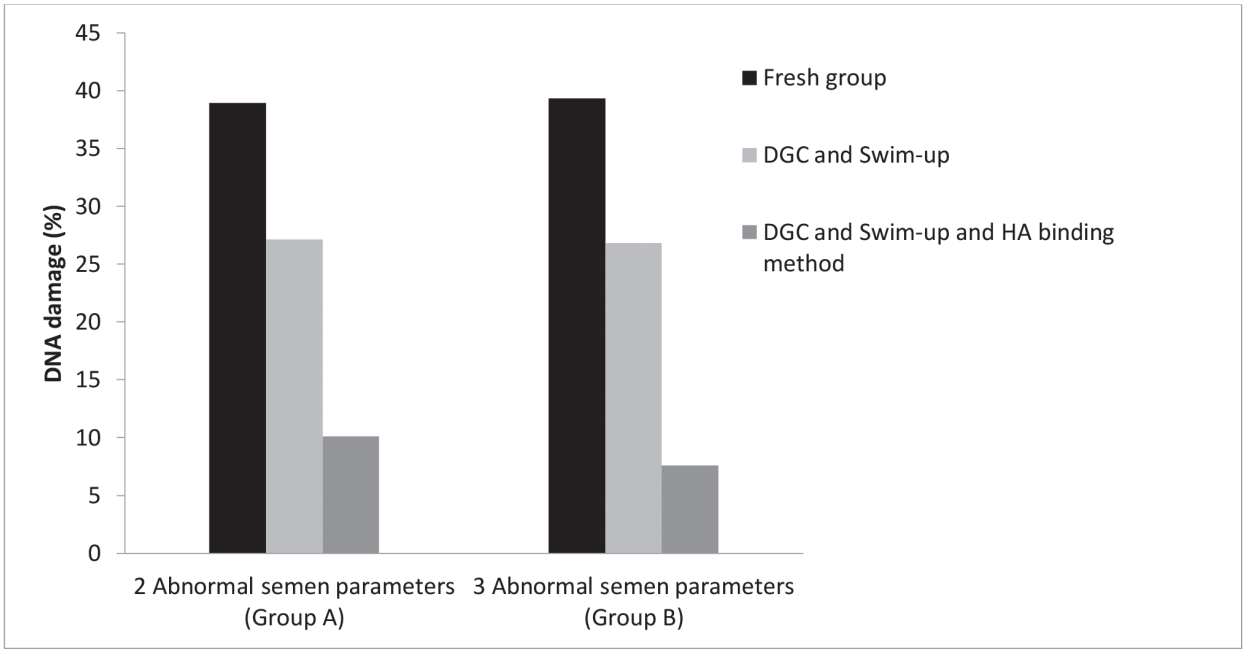


Fig 1. Comparison of DNA damage between conventional method of sperm preparation and additional HBA method in samples with 2 and 3 abnormal semen parameters by TUNEL assay. The DNA fragmentation level in semen containing 2 or 3 abnormalities were similar. After preparation with conventional (DGC and swim up) and additional HBA method, the DNA damage rate was significantly decreased in both groups.

TABLE 2. Univariate analysis for factors of sperm DNA damage using Spearman's rank correlation

Factors	Correlation coefficient (r values)	P value
Age (years)	-0.09	P = 0.51
Abstinent time (days)	-0.01	P = 0.93
Semen volume (mL)	-0.51	P = 0.73
Sperm concentration (million/mL)*	-0.06	P = 0.68
Sperm motility (%)	0.12	P = 0.42
Normal sperm morphology (%)	0.01	P = 0.96
WBC in semen (million/mL)	0.05	P = 0.73

DISCUSSION

Sperm DNA damage influences a series of fertility processes, from fertilization to delivering a healthy baby. Sperm fragmentation may have a negative impact on clinical pregnancy after both IVF and ICSI.[22] Therefore, choosing normal and free of DNA damage sperm for fertilization is a crucial step in creating a new life through assisted reproduction technology. Although sperm motility and morphology are associated with fertilization rate[23, 24] and pregnancy outcome,[25] they are not always representative of the sperm DNA integrity. This study suggests that an additional HBA step following density gradient centrifugation and swim up may help in selecting spermatozoa with lower DNA damage.

Fresh sperm analysis showed higher DNA damage in our study (39%) compared to previous ones, which reported only 6-23%.[4] This may be due to differences in our sample and the reference values used for semen analysis.[4] This study used the WHO 2010[3] cut-off values while the previous study used the WHO 1999 criteria.

Therefore, our study showed more severe abnormalities in semen parameters as lower mean of total motility and normal morphology were applied.

This study also demonstrated the degree of sperm DNA damage post different preparation methods in infertile patients with abnormal semen analysis. Both experimental groups (Group 2 and Group 3) showed reduced sperm DNA fragmentation. To begin with, Group 2 displayed a DNA damage degree of 27% compared to 39% of fresh sample. These results are a consequence of the preparation method itself. To be more specific, most healthy sperm were initially centrifuged to the precipitate and then the most motile sperm swam up to the upper layer of the medium during swim-up process. Thus, most apoptotic sperm were eliminated after conventional preparation. Sperm DNA fragmentation was significantly decreased in Group 3 (9.4%) compared to Group 2 (27%), representing the benefit of an additional HBA step in improving sperm quality. The significant reduction in DNA fragmentation observed in our study after the addition of HBA is consistent with previous studies.[20, 26, 27] DNA damage rates may vary between this study and the former ones, but differences probably originate from the different patient inclusion criteria and different protocols of measuring DNA fragmentation (TUNEL, sperm chromatin dispersion (SCD) test, Chromomycin A3 (CMA3) staining, etc.). For example, Parmegiani[20] studied normozoospermic infertile patients and used the SCD test while we recruited subjects with abnormal semen analysis and used the TUNEL assay.

Regarding sperm DNA damage assessment, there is no consensus on the particular test that offers the most accurate diagnosis to optimize clinical management.[15] The available direct DNA evaluation tests are toxic to sperm and therefore not useful during ICSI. Previous study of our university reported an association of HBA testing with TUNEL assay, and suggested using HBA as an indirect sperm DNA assessment test to select the mature sperm for ICSI.[28] The present study provided even more scientific evidence that HBA reduces DNA fragmentation rate in semen processed with a combination of density gradient, swim up and HBA. Recently, Erberelli reported more than twice higher clinical pregnancy rate by using HBA, although not statistically significant.[18] Therefore, the available literature suggests that HBA selected sperm contain less DNA damage which may lead to better clinical outcome. More clinical research is required to confirm these findings.

In subgroup analysis, no difference in DNA damage rate was found between group A and B containing 2 and 3 abnormal semen parameters, respectively. Therefore, HBA could effectively improve sperm quality in patients with various sperm abnormalities.

We demonstrated that the HA binding process could achieve a three-fold reduction of DNA damage while previous studies demonstrated only two fold.[20] The use of HA binding viscous medium substituted the artificial PVP and had an obvious benefit in providing the selection of spermatozoa with intact DNA at the same time. On the other hand, this protocol could increase the cost of IVF treatment. Nevertheless, there was some limitation in our study. As all the samples were prepared and fixed for TUNEL assay, they could not be used for IVF treatment. Hence, no fertilization rate, embryo development or pregnancy rate could be observed and reported. Further research is needed to study the clinical outcome. Despite, human error may occur while using TUNEL assay because of the similar fluorescent color spectrum. In the future study, the assessment of DNA fragmentation using flow cytometry which is much more objective, should be adopted.

In conclusion, sperm preparation techniques could help decrease DNA damage in spermatozoa. The DNA fragmentation could be subsequently reduced even more by adding the HBA method to the conventional process of sperm preparation. All in all this study suggests that HBA method could be another choice for good sperm selection during ICSI.

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Serum Adiponectin, Visfatin, and Omentin Compared between Non-pregnant and Pregnant Women in Overall, Non-obese, and Obese subjects

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ABSTRACT

Objective: This study aimed to compare serum adiponectin, visfatin, and omentin between non-pregnant and pregnant women in overall, non-obese, and obese subjects.

Methods: There were 40 pregnant and 33 non-pregnant women classified by body mass index (BMI) into non-obese or obese subjects. Fasting blood samples were collected in the morning for the non-pregnant group and before delivery for the pregnant group.

Results: Plasma glucose levels were significantly lower, but plasma insulin levels were significantly higher in pregnant when compared to non-pregnant women in overall, non-obese, and obese women ($p<0.05$ all). The homeostasis model assessment of insulin resistance (HOMA-IR) was significantly higher, but the quantitative insulin sensitivity check index (QUICKI) was significantly lower only in obese pregnant when compared to obese non-pregnant women ($p<0.01$ all). However, in non-obese women, HOMA-IR and QUICKI were comparable between pregnant and non-pregnant women. Serum adiponectin, visfatin, and omentin were significantly lower in pregnant compared to non-pregnant women in overall, non-obese, and obese groups ($p<0.05$ all). In pregnant women, serum adiponectin and omentin levels were significantly lower in obese compared to non-obese pregnant women while serum visfatin levels were comparable in both groups. Serum adiponectin levels were highest followed by omentin and visfatin, respectively in both non-obese and obese pregnant groups. These results indicated that lower serum adiponectin, visfatin, and omentin in pregnant women might contribute to higher insulin resistance in pregnancy. Furthermore, serum adiponectin and omentin were reduced in increasing adiposity similarly to non-pregnant women.

Conclusion: Lower serum adiponectin, visfatin, and omentin in pregnant women might lead to decreased insulin sensitivity in these women.

Keywords: Adiponectin; insulin resistance; omentin; pregnancy; visfatin (Siriraj Med J 2018;70: 219-226)

INTRODUCTION

Pregnancy is a physiological condition that increases insulin secretion to provide sufficient carbohydrates for the growing fetus leading to insulin resistance.[1] In maternal insulin resistance, fat is used more for maternal energy than carbohydrates[1] with a decreased response of target tissues such as liver, adipose tissue, and muscle to insulin.[2] Furthermore, pregnancy is considered as a state of increasing adiposity.[3] Adipose tissue, a powerful endocrine organ, secretes various adipokines which contribute to the regulation of glucose hemostasis and insulin sensitivity including adiponectin, visfatin, and omentin.[4,5]

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Adiponectin, a collagen-like protein, is mainly synthesized by adipocytes and the most abundant adipose-specific protein.[6] It controls the action of insulin by activating AMP-activated protein kinase in the muscle and the liver[7] leading to increased insulin sensitivity.[8] Adiponectin was found to be decreased in type 2 diabetes mellitus patients[9] and was inversely related to insulin levels and the homeostasis model assessment of insulin resistance (HOMA-IR).[5,9] Furthermore, adiponectin levels were decreased in obese compared to non-obese humans[5,10] and were negatively correlated with body mass index (BMI).[11] A previous study found that serum adiponectin levels were significantly lower in pregnant compared to non-pregnant women[12], although another study found no significant difference between pregnant and non-pregnant women when normal and overweight pregnant women were pooled together.[13]

Visfatin, an adipocytokine, was found to be expressed in visceral fat.[14] However, a previous study reported that visfatin expression was found to be comparably expressed in both subcutaneous and visceral adipose tissues in obese subjects, but had a trend to be more expressed in subcutaneous adipose tissue in non-obese subjects.[5] It induces insulin secretion and activates insulin receptor, insulin receptor phosphorylation, and intracellular signaling revealing its insulin-mimetic properties.[15] Administration of glucose to humans caused an increase in circulating visfatin concentrations.[16] Circulating visfatin concentrations were increased in type 1[17] and 2 diabetes mellitus.[17,18] Concentrations of visfatin were found to be lower[10,19], comparable[5], or even higher[20] in obese compared to non-obese subjects. A previous study reported that visfatin concentrations were decreased in non-pregnant women compared to pregnant women even though their pre-pregnancy BMI was matched with non-pregnant women.[21] However, another study reported comparable serum visfatin levels between non-pregnant and pregnant women in the third trimester even though their pre-pregnancy BMI was matched with non-pregnant women.[22] Therefore, the changes of visfatin concentrations were inconclusive regarding obesity or pregnancy.

Omentin is an adipokine mainly secreted from visceral adipose tissue. It increases insulin-stimulated glucose uptake by adipocytes[23] and triggers signaling cascades of an intracellular second messenger, Akt, for multiple cellular functions including glucose metabolism.[24] Levels of serum omentin were lower in impaired glucose tolerance, type 2 diabetes, and obese subjects and were negatively correlated with insulin, HOMA-IR, and BMI.[5,25-27] However, another study found comparable serum omentin between obese and lean subjects.[10] Omentin was found to be lower in normal pregnant women compared to non-pregnant controls without BMI matching.[28]

Collectively, adiponectin, visfatin, and omentin are 3 interesting adipokines that are associated with insulin resistance and obesity. However, comparisons of their serum levels between non-pregnant and pregnant women between groups of their current BMI have not been determined. As BMI/obesity status could affect adiponectin, visfatin and omentin serum levels, and comparisons of their levels within the same BMI group might help to avoid this confounding factor. This study aimed to compare serum adiponectin, visfatin, and omentin levels between pregnant and non-pregnant women categorized into non-obese and obese groups according to their current BMI. Furthermore, the present study also aimed to compare serum adiponectin, visfatin, and omentin between non-obese and obese pregnant women. Comparisons of these adipokines differentiated between non-pregnant and pregnant women classified by BMI could reveal the changes of these peptides without the confounding effect of obesity.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the Siriraj Institutional Review Board (Si.423/2013 for non-pregnant and Si.545/2015 for pregnant subjects) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Informed consent was collected from pregnant and non-pregnant females prior to the study. Sample size calculation for the comparison between the mean of 2 independent groups was obtained as follows: $n = 2 (Z_{\alpha/2} + Z_{\beta})^2/((\mu_1-\mu_2)/\sigma)2$, where $\alpha = 0.05$, 2 sided test, $Z_{\alpha/2} = 1.96$, $\beta = 0.2$, $Z_{\beta} = 0.842$, σ = common SD, $\mu_1-\mu_2$ = difference in mean, and n = sample size per group. Sample size was obtained from previously published papers as 36 per group for adiponectin[12] and 18 per group for visfatin.[22] In this study, there were 40 pregnant and 33 non-pregnant subjects. For non-pregnant women, subjects were recruited from female patients who underwent open abdominal surgery excluding those undergoing endocrine therapy (e.g., steroids, hormone replacement therapy, and thyroxine therapy) and having pregnancy, lactation, traumatic operation, malignancy diseases, operations related to endocrine diseases, severe abdominal inflammation, and menopause. In non-pregnant women, 23 subjects had myoma uteri, 4 subjects had ovarian tumor, 3 subjects had adenomyosis, and 3 subjects had endometriosis. For pregnant patients, subjects who underwent either cesarean or normal labor, had antenatal care and were

in labor at Faculty of Medicine Siriraj Hospital, were aged at least 18 years old, gestational age of at least 34 weeks, and singleton, were recruited. Subjects who had human immunodeficiency virus (HIV) infection, type 1 or type 2 diabetes mellitus, metabolic syndrome, hypertension, other endocrine disorders, previous history of chronic diseases, smoking habits, malignancies, pre-term membranes rupture, fetuses with malformations, and fetal distress during delivery, used drugs that might affect blood glucose and insulin levels, and/or received drugs for pre-term delivery risk, were excluded.

Demographic data of subjects

Age of subjects, gestational age of the pregnant group, and BMI of the non-pregnant group were obtained from questionnaires and medical records. For the pregnant group, their current BMI was calculated from pre-delivery body weight obtained from medical records subtracted by neonatal weight, placental weight, and estimated amniotic fluid weight. The estimated amniotic fluid weight was calculated as 6% of the gestational weight gain obtained from medical record as a previous study reported that amniotic fluid accounted for 6% of the gestational weight gain.[29] BMI classifications for Asians were applied[30] for both pregnant and non-pregnant women into either non-obese (BMI < 23 kg/m^2) or obese (BMI ≥ 25 kg/m^2) groups. In the non-pregnant and pregnant groups, there were 13 and 9 non-obese subjects, respectively and 20 and 31 obese subjects, respectively.

Blood collection

Fasting blood samples were collected in the morning for the non-pregnant group and before delivery for the pregnant group. Blood for glucose and insulin assay was sent to the central laboratory at the Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Some of the samples were stored in clot activator tubes which were immediately kept in an ice bucket and transferred to the laboratory at the Department of Physiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Blood sample was weight balanced and separated by centrifugation at 3000 round per minute (rpm), and stored at 4°C for 15 minutes. Serum sample was stored at -70°C until analysis of adiponectin, visfatin, and omentin.

Glucose and insulin assays

Plasma glucose and insulin were collected during the fasting state and were measured by the central laboratory at the Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. An enzymatic reference method with hexokinase (cobas integra® 800 analyzer) (Roche Diagnostics, Tokyo, Japan) was used for determination of plasma glucose and a sandwich electrochemiluminescent immunoassay (cobas® 8000 modular analyzer series module e602) (Roche Diagnostics, Tokyo, Japan) was used for determination of plasma insulin. Fasting plasma glucose and insulin levels were calculated to determine the levels of insulin resistance using the homeostasis model assessment of insulin resistance (HOMA-IR) which is calculated by multiplication of fasting glucose (mg/dl) and fasting insulin (μU/ml) divided by 405 and the quantitative insulin sensitivity check index (QUICKI) which is calculated by the inverse of the sum of the logarithms of the fasting insulin (μU/ml) and fasting glucose (mg/dl).

Analysis of serum adiponectin, visfatin, and omentin-1 levels

Serum adiponectin levels were analyzed by commercial enzyme linked immunosorbent assay (ELISA) kits (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). The range of adiponectin detection was 0.15-10 ng/ml with a minimum detectable level of 0.15 ng/ml, with 100% cross reactivity to adiponectin (human). Intra-assay variation was 4.73% for pregnant subjects and 2.55% for non-pregnant subjects. Inter-assay variation was 0.92%.

Serum visfatin levels were analyzed using enzyme immune assay (EIA) kits (Phoenix Pharmaceuticals Inc.). The detection range was 0.1–1,000 ng/ml and the minimum detectable level was 1.9 ng/ml with 100% cross reactivity to visfatin C-terminal peptide (human). Intra-assay variation was 4.65% for pregnant subjects and 6.85% for non-pregnant subjects. Inter-assay variation was 6.17%.

Serum omentin-1 levels were evaluated using ELISA kits (Elabscience Biotechnology Co. Ltd., Wuhan, People's Republic of China) with a detection range of 0.63-40 ng/ml, a minimum detectable level of 0.38 ng/ml, cross-reactivity to natural and recombinant HumanITLN1. Intra-assay variation was 3.00% for pregnant subjects and 6.19% for non-pregnant subjects. Inter-assay variation was 2.97%. The absorbance was read at 450 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Statistics

Values are presented as mean ± standard error of mean (SEM) using SPSS 18.0 software. The Kolmogorov-Smirnov test was performed for normality test. For normally distributed data, comparisons between the

pregnant and non-pregnant groups or between the non-obese and obese groups were performed by student's unpaired t-test. For non-normally distributed data, the Mann-Whitney U test was performed. Comparisons between serum levels of adiponectin, visfatin, and omentin in non-obese and obese pregnant women were done using two-way repeated-measures analysis of variance (ANOVA) followed by the post-hoc least significant difference test. Statistical significance for all tests was set at $p < 0.05$.

RESULTS

Demographic and clinical parameters

Clinical parameters of the subjects including age, BMI, plasma levels of glucose and insulin, HOMA-IR, and QUICKI of pregnant women and controls, and gestational age of pregnant women categorized by BMI are shown as mean ± S.E.M in Table 1.

Serum levels of adiponectin, visfatin, and omentin in pregnant and non-pregnant subjects

Serum levels of adiponectin, visfatin, and omentin compared between pregnant and non-pregnant subjects are shown in Fig 1. Serum levels of adiponectin ($p<0.05$ all) (Fig 1A), visfatin ($p<0.01$ all) (Fig 1B), and omentin ($p<0.001$ all) (Fig 1C) were significantly lower in pregnant subjects when compared to non-pregnant subjects in overall, non-obese, and obese groups.

Serum levels of adiponectin, visfatin, and omentin in obese and non-obese pregnant subjects

Serum levels of adiponectin, visfatin, and omentin compared between obese and non-obese pregnant subjects are shown in Table 2. When compared obese to non-obese pregnant women, serum adiponectin and omentin were significantly lower ($p<0.001$), but serum visfatin was unchanged. When compared among serum levels of adiponectin, visfatin, and omentin in non-obese and obese pregnant women, levels of serum adiponectin were highest followed by serum omentin and serum visfatin, respectively in both obese and non-obese subjects ($p<0.001$ all).

TABLE 1. Clinical parameters including age, gestational age, BMI, plasma levels of glucose and insulin, HOMA-IR, and QUICKI of pregnant women and controls categorized by BMI. Values are expressed as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 compared between non-pregnant and pregnant subjects. †p<0.05, ††p<0.01 †††p<0.001 compared with the non-obese pregnant group. Note: For the pregnant group, their current BMI, calculated from pre-delivery body weight obtained from medical record subtracted by neonatal weight, placental weight, and estimated amniotic fluid weight, was used.

Parameters	Overall (n= 73)		Non-obese (n = 22)		Obese (n = 51)	
	Non-pregnant (n = 33)	Pregnant (n = 40)	Non-pregnant (n = 13)	Pregnant (n = 9)	Non-pregnant (n = 20)	Pregnant (n = 31)
Age (year)	44.9±1.3	29.1±0.9***	42.1±2.0	28.4±2.0***	46.8±1.5	29.2±1.0***
Gestational age (week)	-	38.1±0.2	-	37.6±0.2	-	38.3±0.2
BMI (kg/m^2)	25.7±1.2	27.3±0.7	20.5±0.6	21.0±0.5	30.26±1.1	29.2±0.6†††
Glucose (mg/dl)	88.6±3.1	75.3±1.4***	84.9±3.4	70.9±1.4**	91.1±4.7	76.6±1.7**,†
Insulin (μU/ml)	6.4±0.8	12.9±1.2***	4.6±0.9	7.8±1.2*	7.6±1.2	14.5±1.4***,†
HOMA-IR	1.5±0.2	2.5±0.3***	1.0±0.2	1.4±0.2	1.8±0.3	2.8±0.3**,†
QUICKI	0.39±0.01	0.35±0.01**	0.41±0.02	0.37±0.01	0.37±0.01	0.34±0.01**,††

TABLE 2. Serum adiponectin, visfatin, and omentin levels in non-obese and obese pregnant women. Values are expressed as mean ± S.E.M. ***p<0.001 compared between non-obese pregnant and obese pregnant women. †††p<0.001 compared with adiponectin within groups. ‡‡‡p<0.001 compared with visfatin within groups.

Adiponectin (ng/ml)		Visfatin (ng/ml)		Omentin (ng/ml)	
Non-obese	Obese	Non-obese	Obese	Non-obese	Obese
3211.3±396.6	2113.3±199.8***	5.3±1.1†††	4.7±0.6†††	22.5±3.3†††,‡‡‡	12.8±0.9***, †††,‡‡‡

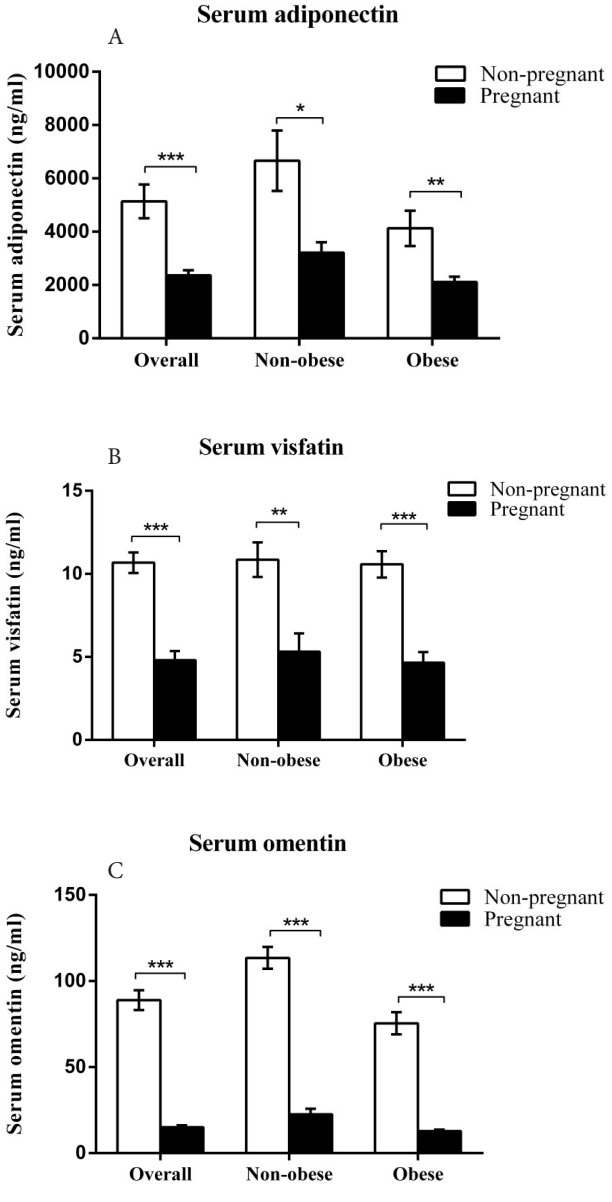


Fig 1. Serum adiponectin, visfatin, and omentin levels compared between pregnant and non-pregnant women in overall, non-obese, and obese subjects. Panels A, B, and C show serum adiponectin, visfatin, and omentin levels, respectively. Values are expressed as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 compared between non-pregnant and pregnant women.

DISCUSSION

This study focused on comparing serum levels of adipokines that are involved in increased insulin sensitivity including adiponectin, visfatin, and omentin in pregnant and non-pregnant women. In pregnancy, there are several physiological changes including increased maternal adiposity and insulin resistance which might lead to changes of these adipokines levels. As a result, in this study, subgroup analysis by BMI classification was performed to decrease confounding effects of obesity from pregnancy.

In this study, plasma glucose levels were significantly lower, but plasma insulin levels were significantly higher in pregnant when compared to non-pregnant women in overall, non-obese, and obese subjects. Blood samples of pregnant women were collected in the latent phase of labor with no strong uterine contraction. As labor is considered as a psychophysiological stress leading to increased corticotrophin releasing hormone and cortisol levels[31], this might interfere with glucose metabolism and/or adipokine levels. Normally, there is a rise of blood glucose levels throughout the labor process[32]; although in this study, blood was drawn during the latent phase of labor which might not lead to a significant increase in glucose levels. Furthermore, glucose levels of pregnant women were lower than non-pregnant women suggesting more nutrients were utilized by neonates that affected the mothers' fasting glucose levels.[33] In pregnancy, insulin-mediated glucose disposal declines by 40–60 %[34] which can lead to insulin resistance.[35] Furthermore, HOMA-IR was higher and QUICKI was lower in pregnancy compared to non-pregnancy, although statistical difference was found only in the obese group. When compared obese pregnant to non-obese pregnant subjects, glucose levels and QUICKI were significantly lower, but insulin levels and HOMA-IR were significantly higher suggestive of marked insulin resistance found in the obese subjects. In obese pregnant women, insulin resistance is amplified by excess lipolysis and release of free fatty acids from expanded

adipose tissue and increased secretion of inflammatory factors (such as IL-6 and TNF-α) and adipokines.[36]

In this study, serum levels of adiponectin, visfatin, and omentin decreased significantly in pregnancy compared to non-pregnancy in the overall, non-obese, and obese groups. For adiponectin, our result had a same trend to a previous study showing that serum adiponectin levels were significantly lower in pregnant (pre-pregnancy BMI <25, gestational age (GA) 10-40 weeks) compared to non-pregnant (BMI <25) women.[12] However, another study found no significant difference between pregnant and non-pregnant women when normal and overweight pregnant women were pooled together.[13] This inconsistency could be explained by the fact that the previous study pooled all weight together, unlike in this study where BMI was classified into obese and non-obese. Obesity status could affect serum adiponectin levels as they were found to be decreased in obese compared to non-obese humans due to the inhibition of adiponectin secretion from high levels of inflammatory cytokines including IL-6 and TNF-α in obese condition.[5] Therefore, lower adiponectin found in pregnant women in both non-obese and obese groups explicitly revealed its low levels in pregnancy regardless of obesity status.

For visfatin, our result was inconsistent with previous studies showing unchanged[22] or even higher[21] visfatin levels in pregnant women even though their pre-pregnancy BMI was matched with non-pregnant controls. These inconsistencies might be because previous studies[21,22] used pre-pregnancy BMI in the pregnant group to match BMI in the non-pregnant group, therefore confounding effects from weight gain in pregnancy, partly from increased adiposity, might affect the levels of visfatin. In this study, BMI of subjects was calculated from their current body weight subtracted by neonatal weight, placental weight, and estimated amniotic fluid weight. Since a previous study found that serum visfatin levels were correlated negatively with weight gain[5], the confounding effect of weight gain in the current study was dissected out. This approach could pinpoint that serum visfatin levels were in fact decreased in pregnancy status.

For omentin, our result was consistent with a previous study showing that omentin levels were lower in normal pregnant women (GA 24-28 weeks) compared to non-pregnant controls without BMI matching.[28] Pregnancy is considered as a state of increased visceral adiposity[37] which is known to decrease omentin levels[5,25] as visceral adipose tissue accretion can cause hepatic toxicity that might inhibit omentin secretion.[38] Collectively, as these adipokines have an effect to increase insulin sensitivity, the decrease in adiponectin, visfatin, and omentin levels in pregnancy might contribute to the cause of insulin resistance to increase blood glucose for the fetus regardless of the obesity status of the mother.

Our study limitation was that age was lower in pregnant women in both obese and non-obese groups. Previous studies reported that adiponectin levels rose with increasing age when male and female subjects were pooled together.[39,40] However, previous studies found that plasma adiponectin concentrations did not change significantly with age in females[40-42] while serum adiponectin levels increased with age in male subjects only.[40] Hence, age does not seem to affect adiponectin levels in women. For visfatin, a previous study revealed no association with age, gender, or BMI.[43] For omentin, previous studies showed either a significant negative correlation[44] or a weak positive correlation[45] between age and omentin levels in newly diagnosed female type 2 diabetic patients and age-matched controls. However, another study found no correlation between serum omentin and age in patients with polycystic ovary syndrome.[46] As a result, the association between omentin levels and age is still inconclusive.

When compared between obese and non-obese pregnant women, serum adiponectin and omentin levels were significantly lower in the obese group while serum visfatin levels were comparable in both groups. These results were consistent with a previous study in non-pregnant women showing low adiponectin and omentin levels, but unchanged visfatin levels in obese compared to non-obese subjects.[5] The decrease in adiponectin and omentin levels in pregnancy indicates that these peptides were reduced in increasing adiposity regardless of pregnancy. Obesity, a state of chronic inflammation with increased levels of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1ß[47], causes the release of free fatty acids and high levels of inflammatory cytokines resulting in inhibition of adiponectin and omentin secretion.[5] For the comparable levels of visfatin between obese and non-obese pregnant women, this result supports the previous study in non-pregnant women that visfatin levels were not affected by the body weight nor BMI.[5,43]

To compare serum adiponectin, visfatin, and omentin in pregnant women, serum adiponectin showed the highest levels in the circulation, followed by omentin and then visfatin, respectively suggesting that adiponectin might have the highest contribution to insulin sensitivity among these three adipokines. These results were in accordance with a previous study in the circulation of non-pregnant women[5] suggestive of similar level patterns of these hormones between pregnant and non-pregnant women.

In conclusion, serum adiponectin, visfatin, and omentin were significantly lower in pregnant compared to non-pregnant women in the overall, non-obese, and obese groups which might contribute to higher insulin resistance in pregnancy. Another study limitation was that the exact amniotic fluid weight could not be obtained. However, the estimated body weight in the pregnancy group in this study is a better representation of current BMI than pre-pregnancy BMI.

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Ultrasonication Extraction and Solid Phase Extraction Clean-up for Quantification of Aristolochic Acid I in the Thai Herbal Antitussive 3 Formula by High Performance Liquid Chromatography with Photodiode Array Detection

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ABSTRACT

Objective: To establish a reliable High Performance Liquid Chromatography method with photodiode array detection (HPLC-PDA) and a suitable extraction method for identification and quantification of Aristolochic acid I (AA-I) in 3 different sample types, i.e. *Aristolochia tagala* Cham.(ATC), Thai Herbal Antitussive 3 Formula (ATF3), and Thai Herbal Antitussive 3 Formula containing ATC (ATF3+ATC).

Methods: Two extraction methods, ultrasonication extraction (UE) and solid phase extraction (SPE), were used in the present study. Reversed phase separations were performed with a SunFire C18 column and HPLC-PDA. Mobile phase was performed by 1 mL/min infusion of mixture of 0.1% o-phosphoric acid and methanol (35:65).

Results: Calibration curve showed a good linearity with R2 equal to 0.9986. Every sample type has been analyzed. While the concentration of AA-I in ATF3 cannot be determined due to the absence of the AA-I peak, the AA-I concentrations in ATC and ATF3+ATC were 7.50-11.34 and 6.78-11.77 mg/mL respectively. In comparison with UE, SPE is more effective in eradication of the interfering compounds and improves the absorbance spectrum of the peak and increases extraction efficiency.

Conclusion: A reliable HPLC-PDA and SPE methods for identification and quantification of AA-I in ATF3+ATC, ATF3, and ATC were established in the present study. The SPE was more effective than UE in the pretreatment step.

Keywords: Aristolochic acid; *Aristolochia tagala* Cham.; Thai Herbal Antitussive 3 Formula; High Performance Liquid Chromatography (HPLC) (Siriraj Med J 2018;70: 227-232)

INTRODUCTION

The widespread occurrence of aristolochic acids (AA) toxicity in Aristolochia plants has greatly restricted their usefulness as dietary supplements or medicines. With the reports that virtually all of plants in genus *Aristolochia* contain AA[1], botanical products containing *Aristolochia* spp are not allowed in many countries including Thailand. In 2011, the National Drug Committee removed Krai-Krue, identified as *Aristolochia pierrei* Lec. and *Aristolochia tagala* Cham. (ATC)[2], from every Thai traditional herbal recipe. Testing for contamination and adulteration of AA in herbal products and medicines is now the focus of attention. These include many Thai traditional herbal recipes in the National Drug list

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previously containing Krai-Krue such as original Ammaruekkhawatee, Homnawakod, Homintrajak, and Thartbanjob. The Herbal Antitussive 3 Formula (ATF3), used in this study, contains the herbal ingredient as Ammaruekkhawatee. ATF3 has been used to relieve cough and reduce mucus in respiratory syndrome patients. It contains six herbal components which are *Terminalia chebula* Retz., *Glycyrrhiza glabra* L., *Cuminum cyminum* L., *Coriandrum sativum* L., *Phyllanthus emblica* L., and *Terminalia bellirica* (Gaertn.) Roxb.

AA is a mixture of structurally related nitrophenanthrene carboxylic acids compounds. AA and its derivatives are known to be nephrotoxic[3-7] and carcinogenic agents.[6-9] Aristolochic acid I (AA-I) is the most abundant of these compounds.[10] Although many assays can detect AA-I, a procedure based on High Performance Liquid Chromatography with photodiode array detector (HPLC-PDA) is a feasible choice, because of its ease of use and low cost of operation and maintenance, especially when compared to the other hyphenation procedures such as Liquid Chromatography with Mass Spectrometry (LC-MS). Previous studies revealed many extraction methods for detection of AA-I from Aristolochia plants such as ultrasonication extraction (UE)[11-16], Solid phase extraction (SPE)[17-18], Soxhlet extraction[19] and other techniques.[19,20] Among these, UE is an easy and efficient method for AA-I quantification when involving single herbal components. However, with complex herbal recipes, multiple peak separation still remains a significant problem. Concurrently, SPE has demonstrated efficacy when used in crude drugs and Kampo fomulations.[17] Therefore, testing of such SPE condition is needed in order to demonstrate similar efficacy for Krai-Krue containing ATF3 (ATF3+ATC). The aim of this study was to establish a reliable HPLC-PDA and a suitable extraction method for identification and quantification of AA-I in 3 different sample types, i.e. ATC, ATF3, and ATF3+ATC.

MATERIALS AND METHODS

Materials and solid phase

All plant materials were prepared by the GMP certified Manufacturing Unit of Herbal Medicine and Products, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Krai-Krue used in the present study was identified as *Aristolochia tagala* Cham. (ATC). Three batches of herbs were used, with each batch comprising of 3 sample types, ATC, ATF3, and ATF3+ATC. HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from LEDA (Spain). Analytical grade o-phosphoric acid was obtained from Merck (Germany). Standard AA-I was purchased from Sigma Chemical Co. (St. Louis, USA). HPLC quality water was purified using a Milli-Q water system from Millipore (France). Sep-Pak Vac 1cc (100 mg) Waters Accell Plus QMA cartridges were used.

Instrument and HPLC condition

Analyses were carried out using a Waters Alliance 2695 HPLC system with a 2998 photodiode array (PDA) detector and Empower Software. The separations were performed on a reverse phase column (Sunfire C18, 5µm, 4.6x150 mm, Water, Milford, MA), maintained at 25°C. The various percentages of mobile phase comprising of MeOH and 0.1% o-phosphoric acid were observed to optimize the separation and sensitivity. The experiments were performed under isocratic elution which consisted of 0.1% o-phosphoric acid: MeOH at 35:65 (v/v) at 1 mL/min flow rate. The injection volume for all samples was 10 µL. The PDA detector was scanned from 200 to 450 nm. The quantification wavelength was set at 393 nm to reduce interferences by other constituents.

Extraction of samples

According to the original formula of ATF3+ATC, there was 3.292 mg of ATC per 40 mg of ATF3+ATC. Therefore, the quantification of AA-I was performed with 3.292 mg/mL of ATC and 40 mg/mL of ATF3+ATC in order to maintain equal amounts of ATC in these 2 sample types.

Ultrasonication extraction (UE)

ATF3+ATC batch 1 and ATC batch 1 were used to optimize the number of UE. A sample from batch 1 of each sample type was divided into 3 groups which went through single, double and triple extraction cycles with 30 minutes ultrasonication for each extraction cycle in 100% MeOH. Supernates were separated from precipitates by centrifugation at 6,500 rpm for 10 minutes at 4˚C and filtered through the 0.2 µm PVDF (polyvinylidene fluoride) membrane before injection into the HPLC. The concentrations of AA-I for samples ATF3+ ATC and ATC alone are shown in Fig 1. For both sample types, double and triple UE yielded statistically higher concentration of AA-I compared to single extraction (Fig 1). However, when comparing double versus triple extraction, no differences in the yield of AA-I were seen. Therefore, the quantification experiment proceeded with only double extraction. (Table 1)

Solid phase extraction (SPE)

The SPE condition from Yamasaki K *et al*[17] was adapted, in order to obtain the appropriate procedure

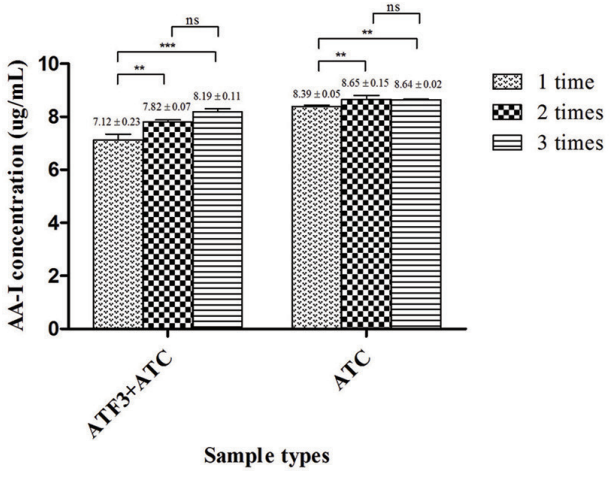


Fig 1. Test for the number of ultrasonication. The result expressed as mean±SD of triplicate experiments. One-way ANOVA followed by Bonferroni's *post hoc* test were applied for statistical study. ns = not significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

based on ATF3. First, all sample powders were extracted twice by mixing with 1% ammonia in MeOH over 10 minutes. Supernatants were separated from precipitates by centrifugation at 6,500 rpm for 10 minutes at 25°C. One milliliter of extraction solution was loaded onto the QMA cartridge, which was previously equilibrated with 1 mL of 1% ammonia in MeOH. In this step, ammonia ionized AA-I to negatively charged ion and adhered with an anion exchange resin. Next, washing was performed with 5 mL of MeOH in order to remove the interfering peaks which were seen to immediately precede the AA-I peak. The washing was done until the interfering peaks disappeared. Finally, cartridges were eluted with 1 mL of ACN:water:o-phosphoric acid (80:20:2) according to the best elution yield compared with other mixed ratios of ACN:water:o-phosphoric acid for elution solution. Each eluent was filtered through the 0.2 µm PVDF membrane before the analysis.

Validation of the HPLC method

Stock solution of standard AA-I (100 µg/mL) was prepared by accurately weighing of standard AA-I and dissolving in MeOH using a volumetric flask. After filtering through 0.2 µm PVDF membrane filters, working standard solutions were prepared by further dilution of the stock solution with the appropriate volume of MeOH. Each concentration was analyzed in triplicate. The linearity was evaluated by analysis of the standard curve.

The method precision was evaluated from relative standard deviation (RSD) of the area under the curve of each standard concentration. Three standard concentration solutions were performed five times in one single day for intra-day precision. For inter-day precision, the measurements from three standard concentrations solutions were performed three times a day on three consecutive days.

Recovery of the method was performed by adding of three known amounts of the standard solution into the selected samples. Since, the chromatogram of UE of ATF3 batch 2 and 3 (Fig 3B, 3C) showed similar patterns which contained the interfering peak preceding that of AA-I peak. In order to test whether or not it effected the recovery of the method, ATC and ATF3 batch 2 were selected as the representatives for the recovery test of ATC and ATF3, respectively.

Statistical analysis

The concentrations of AA-I in different sample types

TABLE 1. Concentration of Aristolochic acid I in ATC and ATF3+ATC. The result expressed as mean±SD of six values. Two-way ANOVA followed by Bonferroni's *post hoc* test was applied for statistical study. ND = not determine, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Sample type	Batch	AA-I concentration (µg/mL)		P-value
		UE	SPE	
ATC	1	8.02±0.08	11.34±0.11	<0.001
	2	7.50±0.09	8.51±0.18	<0.001
	3	8.64±0.27	9.66±0.32	<0.001
ATF3+ATC	1	6.78±0.05	9.81±0.12	<0.001
	2	ND	6.94±0.30	-
	3	ND	11.77±0.51	-

and through various extractions were represented as mean±standard deviation of 3 and 6 measurements. One-way and two-way analysis of variance with Bonferroni post *hoc*-test were performed. This software used GraphPad Prism™ version 5.02 for Windows (GraphPad Software, www.graphpad.com); a *p*-value of less than 0.05 was considered to be significant.

RESULTS

The results of satisfactory precision and recovery rates were shown in Tables 2 and 3. The precision RSDs were 0.60-3.30 for intra-day and 2.15-3.83 for inter-day (Table 2). The recovery of AA-I of ATC and ATF3 were in range of 93.73-103.27%. Whereas, the recovery result from UE of ATF3 was not reported in the present study (Table 3) because of the interfering peak preceding that of AA-I peak after addition of AA-I standard solution. The calibration curve was investigated in the range of 2-18 µg/mL (2, 6, 10, 14, 16, 18 µg/mL). The regression equation and correlation coefficient (R^2) were as follows: $Y = 14408.942\ X - 6893.590$ and $R^2 = 0.9986$, respectively. Limit of detection (LOD) and limit of quantification (LOQ), calculated as the concentrations of AA-I needed to produce signal-to-noise ratios (S/N) of 3 and 10 were determined to be 0.7 and 2.0 µg/mL, respectively. All ATC samples from both UE and SPE methods represented similar chromatograms and UV spectrum patterns as ATC sample from batch 1 (Fig 2B and 2b). Based on retention times and UV spectra patterns, AA-I was found in all ATC samples. In contrast, the concentration of AA-I could not be determined due to the absence of the AA-I peak in all UE of ATF3 samples (Fig 2C-E). Therefore, the SPE experiment for all ATF3 samples were not needed to be performed and the amounts of AA-I in all ATF3 samples were not reported (Table 1). When compared to UE (Fig 3a-c), the absorbance spectrum of ATF3+ATC batch 1-3 using SPE (Fig 3d-f) showed more similar pattern to standard AA-I. (Fig 2a). SPE improved the absorbance spectrum of the peak in this case. UE of ATF3+ATC batches 2 and 3 (Fig 3B, 3C) showed interfering peaks which could not be distinguished from AA-I peaks, which lead to incorrect peak areas and amount calculation. In these particular cases, AA-I concentrations were reported as not determined (ND) as shown in Table 1. While, the SPEs of ATF3+ATC batches 2 and 3 (Fig 3E, 3F) show a valuable clean-up of the problematic peaks resulting in a proper quantification of AA-I.

TABLE 2. Intra-day and inter-day precisions of Aristolochic acid I quantification.

Chemical compound	Concentration (µg/mL)	Intra-day (RSD)	Inter-day (RSD)
Aristolochic acid I	2	3.30	3.83
	10	2.64	3.28
	18	0.60	2.15

TABLE 3. Recovery tests of Aristolochic acid I in UE of ATC batch 2, SPE of ATC batch 2, and SPE of ATF3 batch 2. Each concentration was performed in triplicate.

Sample	Extraction method	Amount added (µg/mL)	Amount measured (µg/mL)	% recovery	Mean % recovery
ATC	UE	6.00	5.56	92.66	93.73±1.90
		7.50	7.05	93.98	
		9.00	8.51	94.54	
ATC	SPE	6.80	6.38	93.76	103.27±7.53
		8.50	9.06	106.62	
		10.20	11.16	109.44	
ATF3	SPE	5.50	5.70	103.67	100.59±3.83
		6.90	7.00	101.47	
		8.30	8.02	96.63	

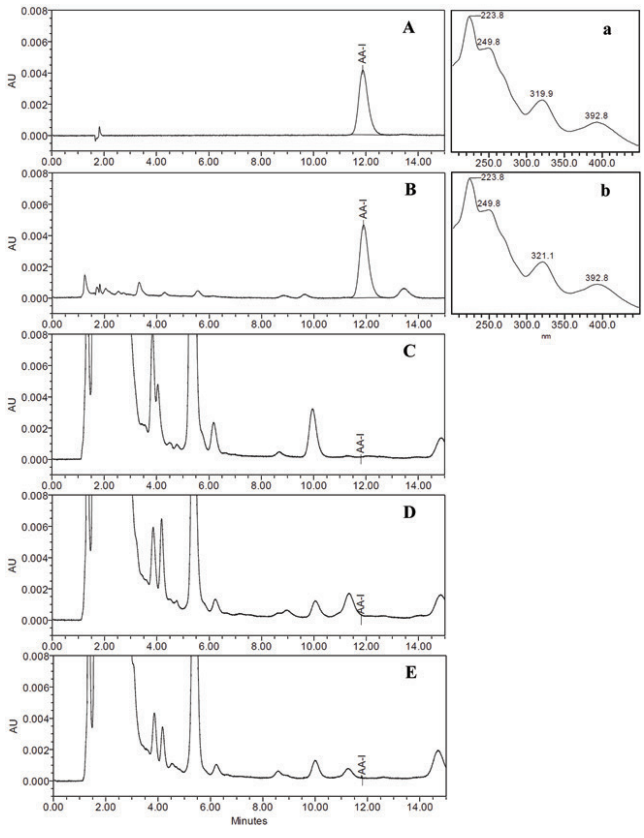


Fig 2. HPLC chromatograms (393 nm absorbance) of **(A)** standard AA-I; **(B)** UE of ATC batch 1; **(C)** UE of ATF3 batch 1; **(D)** UE of ATF3 batch 2; **(E)** UE of ATF3 batch 3; (a) Absorbance spectrum of standard AA-I recorded at 200-450 nm; (b) absorbance spectrum of the AA-I peak in UE of ATC batch 1.

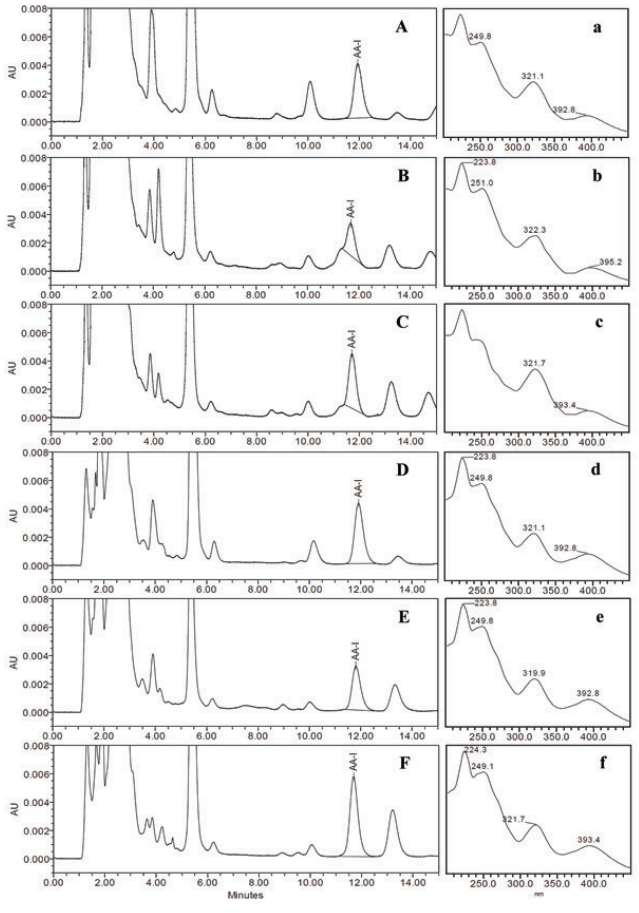


Fig 3. HPLC chromatograms (393 nm absorbance) of **(A)** UE of ATF3+ATC batch 1; **(B)** UE of ATF3+ATC batch 2; **(C)** UE of ATF3+ATC batch 3; **(D)** SPE of ATF3+ATC batch 1; **(E)** SPE of ATF3+ATC batch 2; **(F)** SPE of ATF3+ATC batch 3 **(a)**; Absorbance spectrum of the AA-I peak in UE of ATF3+ATC batch 1; **(b)** absorbance spectrum of the AA-I peak in UE of ATF3+ATC batch 2; **(c)** absorbance spectrum of the AA-I peak in UE of ATF3+ATC batch 3; **(d)** absorbance spectrum of the AA-I peak in SPE of ATF3+ATC batch 1; **(e)** absorbance spectrum of the AA-I peak in SPE of ATF3+ATC batch 2; **(f)** absorbance spectrum of the AA-I peak in SPE of ATF3+ATC batch 3.

DISCUSSION

There is no doubt that different batches of herbs can contain varying types and amount of compounds even in the same species of plant. A wide range of factors can modulate the active components of herb products, including physiological, environmental/cultural factors and genetic variation.[21] Therefore, the different chromatographic patterns from UE of ATF3+ATC batch 1 compared with batches 2 and 3 were not entirely unexpected. In addition, the good values of recovery from SPE of ATF3+ATC (Table 3) represented that SPE eliminated the interfering peaks without effect recovery of the method. When compared with other studies, there were 2 new findings in this study. Firstly, SPE showed a significant increase in AA-I concentrations compared to UE for all samples. This confirmed that SPE is a more efficient extraction method than UE alone. Secondly, it can be assumed that AA-I originates from the ATC component, since AA-I was found in ATF3+ATC, but not in ATF3 alone. In addition, the difference of AA-I concentrations in ATC compared with ATF3+ATC using SPE within the same batch indicated that the differences in type and amount of compound integrands in ATF3 in the sample matrix affected the efficiency of extraction. Overall, the ability of SPE to eradicate interfering compounds while improving the absorbance spectrum of AA-I peak and extraction efficiency makes it a more superior method for sample extraction of complex herbal mixtures.

CONCLUSION

This study provides reliable clarification method for the use of HPLC-PDA in analyzing AA-I compound in complex herbal mixtures. Since eradication of interfering peaks is crucial for accurate AA-I quantification using HPLC-PDA, the understanding of the role of SPE may contribute to the development of future methods for analyzing other complex herbal mixtures.

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Obstetric and Perinatal Outcomes of Singleton Pregnancies After in-vitro Fertilization in Siriraj Hospital, A Matched Case-Control Study

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ABSTRACT

Objective: To compare obstetric and perinatal outcomes between singletons born after IVF and natural conception.

Methods: A total of 141 singleton pregnancies conceived by IVF were included. Another 141 singleton pregnancies conceived naturally were randomly selected as comparison group, matched by age. Data were retrospectively extracted from medical records, including baseline characteristics and delivery data. Various characteristics, including pregnancy and neonatal outcomes were compared between groups.

Results: Women in IVF group were more likely to be nulliparous and had previous miscarriage (88.7% vs. 76.6%, 0=0.003; and 26.2% vs. 14.8%, p=0.018, respectively). Underlying diseases and complications during pregnancy were comparable between the 2 groups. Mean gestational age was lower in IVF group (37.9 ± 2.0 vs. 38.4 ± 1.6, p=0.008), but without clinical significance. Primary cesarean section was significantly more common among women in IVF group (74.4% vs. 54.6%, p<0.001) and the majority were elective cases (61.9% vs. 23.4%, p<0.001). There were no significant differences in terms of rates of preterm labor, birth weight, low birth weight, small for gestational age, neonatal intensive care unit admissions, and perinatal mortality.

Conclusion: Singleton pregnancies after IVF were not associated with higher risks of adverse obstetric and perinatal outcomes, compared with naturally conceived group, but IVF pregnancies are associated with a high rate of cesarean sections.

Keywords: IVF; Natural conception; pregnancy outcomes; neonatal outcomes; cesarean (Siriraj Med J 2018; 70: 233-237)

INTRODUCTION

Since the first successful birth after in vitro fertilization and embryo transfer (IVF/ET) reported in England in 1978, the number of babies resulting from the assisted reproductive technology (ART) has increased all over the world.[1] At first, IVF was developed for the woman with tubal problem (blocked or damaged fallopian tube), but now it is the treatment of choice for other causes of infertility that are refractory to more conservative treatment.[2,3] Although most pregnancies after IVF result in normal and good outcomes, many studies reported the higher rates of the cesarean section, preterm birth, low birth weight (LBW) and poor perinatal outcomes compared with general population.[4]

Recently, there were a great number of women that had a tendency to be married and desire for pregnancy at older age. The infertility rate among that group seems to have increased. This, in turn, raises the demand for

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infertility treatment especially assisted reproduction technology. Maternal factors related to infertility are also independently associated with adverse obstetrical outcomes. For example, advancing maternal age is associated with both declining fertility and multiple adverse outcomes of ongoing pregnancy.[5] A higher incidence of obstetric complications of advanced maternal age pregnancies, including pregnancy induced hypertension (PIH), gestational diabetes (GDM), placenta previa and abnormal labor patterns, has been reported.[6,7] This observation is likely related to the underlying progressive vascular endothelial damage that occurs with aging.[7] This is of lesser parity than the average gravida, so IVF pregnancies are more likely to deliver by cesarean section.[8]

In Siriraj Hospital, the rate of IVF treatment has increased year by year. Obstetric and perinatal outcomes of the IVF pregnancies, regarding the association with adverse outcomes, are always of concern. The aim of our study was to compare obstetric and perinatal outcomes between singletons born after IVF and natural conception.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of Siriraj Hospital (Si 515/2014). 141 singleton pregnancies conceived naturally or by IVF from July 2006 to December 2013, and delivered at Siriraj Hospital were enrolled. In IVF pregnancy group, the indication for IVF included male factor, tubal factor, and peritoneal factor (e.g. pelvic endometriosis, pelvic adhesion). The analysis included only pregnancies leading to live births (less than 500 grams of birth weight).

The IVF and naturally conceived group were matched 1:1 for age (±2 years) and time of delivery (within same day). They were treated by the same obstetric service. All the data regarding the examinations, hospitalization, delivery and discharge were kept in the standardized medical records. All newborns received physical examination after birth and all abnormalities were noted. The gestational age of IVF group was calculated by adding 14 days to the day of the oocyte retrieval; while for the spontaneous conception, it was estimated based on the last menstrual period or the first ultrasound results. Retrospective chart review was performed. Data of the maternal complications during antenatal and intrapartum period, gestational age, mode of delivery, perinatal mortality and neonatal outcomes were collected.

Data were analyzed using SPSS ver. 17.0 (An IBM Company). The statistical significance of differences was assessed by chi-square test and unpaired t-test. A p-value of < 0.05 was considered statistically significant.

RESULTS

Maternal characteristics

The mean maternal age was similar in both groups (Table 1). The frequency of nulliparity in the IVF group was significantly higher than that in the spontaneous conceived group (88.7% vs. 76.6%, P = 0.015). Poor outcome of previous pregnancy was more frequent in the IVF group (P = 0.018). There were no significant differences in pre-gestational underlying disease.

Obstetric complications

There were no statistically significant differences in the obstetric complications between the IVF and the spontaneous conceived groups (Table 2). The rates of GDM, PIH, anemia, preterm labour, preterm PROM and intrauterine growth retardation (IUGR) were similar in both groups.

Labor and delivery characteristics

Mean gestational age at delivery was significantly lower in IVF group (37.9 ± 2.0 vs. 38.4 ± 1.6; P = 0.008), although there was no clinical significance. Normal vaginal delivery was found in significantly fewer patients in the IVF group. This was attributed mainly to the significantly higher primary cesarean section, as well as the elective cesarean section rate, in the IVF group compared with the control group (Table 3).

Neonatal outcome

No differences were noted between the IVF and spontaneous conceived groups in terms of mean birth weight, the incidence of very low birth weight (< 1,500 g) and low birth weight (1,500-2,500 g) (Table 4). The frequencies of SGA, asphyxia and neonatal intensive care admissions were also not significantly different.

DISCUSSION

The present study showed the IVF outcomes of singleton pregnancy compared with natural conceived baby in Siriraj Hospital, Thailand. The results in the study revealed that IVF pregnancies were mostly primiparous and have history of a poor pregnancy outcome as shown in previously reports.[4,9,10] These characteristics were associated with obstetric risk and adverse outcome leading to the explanation of the different outcome between IVF groups and natural conceived groups.[11] Several literatures have reported controversial data regarding the rate of obstetric complications during pregnancies.[12,13] Some studies that have similar findings showed that IVF pregnancies did not increase risk for most obstetric complications such

TABLE 1. Maternal characteristics between IVF and Spontaneous pregnancies.

Characteristics	IVF (N=141)	Spontaneous (N=141)	P value
Mean maternal age ± SD (years)	34.5 ± 3.6	34.1 ± 3.6	0.400
Nulliparous	125 (88.7%)	108 (76.6%)	0.015
Outcome of previous pregnancy			
Miscarriage	37 (26.2%)	21 (14.9%)	0.018
Pre-gestational underlying disease			
Chronic hypertension	3 (2.1%)	3 (2.1%)	1.000
Pre-gestational DM	0 (0%)	2 (1.4%)	0.156

TABLE 2. Antepartum complications between IVF and spontaneous pregnancies.

Characteristics	IVF (N=141)	Spontaneous (N=141)	P value
GDM			
GDMA1	11 (7.8%)	9 (6.4%)	0.643
GDMA2	1 (0.7%)	2 (1.4%)	0.562
Pregnancy-induced hypertension			
Gestational hypertension	4 (2.8%)	1 (0.7%)	0.176
Preeclampsia	1 (0.7%)	1 (0.7%)	1.00
Anemia	0 (0%)	1 (0.7%)	0.316
Preterm labor	16 (11.3%)	14 (9.9%)	0.699
Preterm PROM	4 (2.8%)	6 (4.3%)	0.520
IUGR	4 (2.8%)	1 (0.7%)	0.176

TABLE 3. Labor and delivery characteristics between IVF and spontaneous pregnancies.

Characteristics	IVF (N=141)	Spontaneous (N=141)	P value
Mean gestational age at delivery ± SD (weeks)	37.9 ± 2.0	38.4 ± 1.6	0.008
Gestational age at delivery			
< 34 weeks	6 (4.3%)	2 (1.4%)	0.333
34 – 36 weeks	10 (7.1%)	12 (8.5%)	
≥ 37 weeks	125 (88.7%)	127 (90.1%)	
Route of delivery			
Vaginal delivery	18 (12.8%)	53 (37.6%)	<0.001
Repeat cesarean section	18 (12.8%)	11 (7.8%)	0.085
Primary cesarean section	105 (74.4%)	65/105 (61.9%)	<0.001
Elective cesarean section	77 (54.6%)	18/77 (23.4%)	<0.001
Postpartum hemorrhage	4 (2.8%)	2 (1.4%)	0.684

TABLE 4. Neonatal outcomes between IVF and spontaneous pregnancies.

Characteristics	IVF (N=141)	Spontaneous (N=141)	P value
Mean birth weight ± SD (g)	3,008.6 ± 571.5	3,030.5 ± 452.7	0.721
Birth weight			
<1,500 g	3 (2.1%)	0 (0%)	0.145
1,500-2,500 g	14 (9.9%)	10 (7.1%)	
>2,500 g	124 (87.9%)	131 (92.9%)	
SGA	5 (3.5%)	2 (1.4%)	0.447
Birth asphyxia	1 (0.7%)	2 (1.4%)	0.562
NICU admission	5 (3.5%)	6 (4.3%)	0.758
Sex ratio (Male:Female)	1.01	0.99	0.905

as PIH, GDM, preterm birth, PPROM and IUGR.[12-15] This may be due to our matching method and the same treatment protocols in the university hospital. On the other hand, there is a study which found that various gestational complications occurred more frequently in IVF group.[16] One can speculate that the intense surveillance and careful monitoring of IVF pregnancies may have resulted in the higher rate of diagnosis of complications, whereas it remained undetected among spontaneous conceived group.

The authors found that the rate of preterm delivery, low birth weight and perinatal outcomes in IVF and spontaneous conceived groups were similar, and the results of preterm delivery rate and low birth rate in the study were compatible with previous study of preterm delivery in Siriraj Hospital (9-13%) and low birth weight rate of Thailand (8.1%).[17] This was opposed to the research which reported the increased prematurity and low birth weight after IVF compared to the general population.[12,14,18] However, there was also a study which was consistent with our results.[9] In that detailed analysis, it revealed that the trend toward an increased prematurity rate after conceived IVF mainly resulted from a non-significantly increased incidence of preterm cesarean sections, rather than premature spontaneous deliveries. Various obstetric complications were managed in IVF group by cesarean sections in the preterm period.[19] In Siriraj Hospital, the survival rate of premature infants has increased due to the establishment of obstetric and newborn care team including excellent equipment and well-trained neonatologists which result in the good outcome of premature newborns. This, therefore, was a possible explanation of similar perinatal outcomes of both groups. However, there were 2.1% of VLBW in the IVF group while there was no incidence of VLBW in the spontaneous conceived group. Although the number of SGA and IUGR were not significantly different between the groups, the frequencies of IUGR and SGA in IVF groups were 4 and 2-fold higher than that of the naturally conceived groups, respectively. A larger sample size may be required in future study to clarify these.

The incidence of cesarean section was higher in the IVF group than in the comparison group. Similar results have been reported in many studies.[20,13] Although the repeated cesarean section was excluded, the rate of primary cesarean section remained statistically significantly higher among the IVF pregnancies compared with spontaneous conceived pregnancies. The higher frequency in the IVF group is explained by the increase in elective cesarean section due to minimized maternal complication during the late term pregnancy or post term pregnancy. The high cesarean section rate in spontaneous conceived pregnancies (about 31% excluded elective C/S) was found with uncertain reason. Nevertheless, it is consistent with previous study that reported a trend in methods of delivery[21] and is now far beyond the rate suggested by WHO.

There are advantages of this study, the obstetric outcome of IVF patients from a single unit was compared with that of spontaneous conceived group with matching and included only singleton pregnancies to avoid the possible confounding effects of multiple pregnancies.

The limitations of the study are small sample size and we cannot record all data of every IVF patient because they have delivered in another hospital.

In conclusion, after matching the maternal age, location and date of delivery, the risk of preterm birth, low birth weight and poor perinatal outcomes, as well as obstetric complication were not associated with IVF. However, there were some poorer outcomes in the IVF group. Thus, may require a larger sample size and further research to evaluate the factors that affect the outcome of babies born after IVF.

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Development of a Novel Method for Determination of 11 Antiretroviral Drugs Concentrations in Human Plasma by Ultra-Performance Liquid Chromatography-Photodiode Array Detection Method

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ABSTRACT

Objective: To develop ultra-performance liquid chromatography-photodiode array detection (UPLC-PDA) method that is fully validated for measuring 11 antiretroviral drugs concentrations in human plasma.

Methods: Chromatographic separation was performed on an ACQUITY UPLC™ BEH Shield RP, 1.7 µm (100 mm x 2.1 mm I.D.) and used acetronitrile with 0.1% formic acid in Milli-Q water as a mobile phase. ACQUITY UPLC® Photodiode Array (PDA) Detector was performed at 210, 240, and 260 nm.

Results: This method demonstrated a good separation result for plasma levels of 11 antiretroviral drugs within 16 minutes. The lower limit of quantification (LLOQ) was 0.25 µg/mL for maraviroc, 0.5 µg/mL for lopinavir, and 20 ng/mL for the remaining 9 antiretroviral drugs. This method was fully validated in terms of selectivity, accuracy, precision, and stability. The standard curves are in the expected ranges of drug concentration in the patients' plasma with good linearity (r2>0.995). Recoveries of extraction ranged from 72.27- 110.80% with repeatability.

Conclusion: A novel, sensitive, accurate, and reproducible UPLC/PDA method for quantification of 11 currently-prescribed antiretroviral drugs concentrations in human plasma was successfully developed and fully validated according to USFDA guidelines.

Keywords: UPLC/PDA; antiretroviral; therapeutic drug monitoring (Siriraj Med J 2018;70: 238-246)

INTRODUCTION

Antiretroviral (ARV) drugs that are commonly used to treat human immunodeficiency virus (HIV) include: protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), integrase inhibitors (INSIs), and entry and fusion inhibitors (EIs). The use of three or more antiretroviral medicines is referred to as highly active antiretroviral therapy or HAART. Presently, HAART is the standard treatment for HIV infection.[1]

Failure of treatment with antiretroviral drugs in HIV patients has remained unacceptably high. Low concentrations of ARVs are evaluated as drug administration's failure. High concentrations of ARVs also increase the risk of drug toxicity.[2] Recommended standard dosages of multi-drug ARVs in patients with immune deficiencies who have some degree of liver and/or kidney impairment may be dangerously higher than the general regimen. Therapeutic drug monitoring (TDM) can identify and prevent excessively abnormal ARV concentration. This can increase clinical response and decrease their overdose's toxic side effect. The target therapeutic concentrations in the leading ARVs are, as follows: atazanavir >200 ng/mL;

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lopinavir >1,000 ng/mL; ritonavir >2,100 ng/mL; efavirenze >1,000 ng/mL; nevirapine >3,500 ng/mL; maraviroc >50 ng/mL; darunavir >3,300 ng/mL; etravirine >300 ng/mL; raltegravir >65 ng/mL; elvitegravir >200 ng/mL; and, dolutegravir >40ng/mL.[2-4]

Many methods for the simultaneous quantification of ARVs have been developed. The most commonly used techniques are liquid chromatography-tandem mass spectrometry (LC-MS/MS)[5-11] and reversed-phase HPLC with UV detection.[12-21] The LC-MS/MS tandem method is not only highly sensitive and highly specific, but it also has a very short runtime, because complete separation of all antiretroviral's chromatogram is not necessary. However, the limitation of LC-MS/MS analysis is not only requiring a more expensive instrument than other techniques, but also susceptibility to their matrix effects. Moreover, HPLC methods with UV detection have a very long analytic time to complete the resolution of all chromatographic peaks. High sensitivity, high specificity, and short runtime are ideal performance parameters of therapeutic drug monitoring (TDM). Then, Ultra-performance liquid chromatography (UPLC) may have an opportunity in this TDM because of its advantages of cheaper method and can detect broader separated chromatograms. Although other methods for the simultaneous quantification of protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) using UPLC with photo diode array detection have been published[22,23], neither of those studies included the quantification of integrase inhibitors (INSIs), and entry and fusion inhibitors (EIs).

The aim of this study was to develop the ultra-performance liquid chromatography-photodiode array detection (UPLC-PDA) method which has been fully validated for measuring 11 antiretroviral concentrations in human plasma. We anticipate that this method will have clinical value for monitoring the levels of these drugs in patients who received several antiretroviral drugs' administration.

MATERIALS AND METHODS

Chemicals and Materials

Atazanavir, etravirine, raltegravir, elvitegravir, and dolutegravir were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Maraviroc, lopinavir, ritonavir, darunavir, efavirenze, nevirapine, and tenofovir (internal standard, IS) were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany). HPLC grade acetonitrile and methanol were purchased from Labscan Ltd. (Bangkok, Thailand). Water used for experimentation was produced by Milli-Q® water purification system (EMD Millipore, Billerica, MA, USA). Any other chemicals used were of analytical grade. Drug-free human plasma was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital. The protocol for this study was approved by the Siriraj Institutional Review Board (Si 655/2015), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Instruments and Analytical Conditions

Acquity™ Ultra Performance Liquid Chromatography (Waters Corporation, Milford, MA, USA) was used for the separation module. Chromatographic separation was performed on an ACQUITY UPLC™ BEH Shield RP (1.7 µm, 100 mm x 2.1 mm I.D.) (Waters Corporation, Milford, MA, USA). To achieve an optimum result, the mobile phase was performed with a flow rate of 0.45 mL/min and a column temperature of 40°C. The gradient programmed for the mobile phase was optimized using acetronitrile and 0.1% formic acid in water, as shown in Table 1. ACQUITY UPLC® Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA) was operated at wavelengths between 200-380 nm and quantitation was performed at 210 nm for maraviroc and lopinavir; 240 nm for nevirapine, raltegravir, atazanavir, ritonavir, efavirenze, and etravirine; and, 260 nm for dolutegravir, darunavir, and elvitegravir. The autosampler was conditioned at 8°C and the injection volume was 10 µL. Empower 2 software (Waters Corporation, Milford, MA, USA) was used for data management.

Preparations of Standard and Quality Control Samples

Standard stock solutions of both analytes and IS were prepared in dimethyl sulfoxide at a concentration of 400 µg/mL. Stock solutions were diluted with a mixture of methanol and water (50:50, v/v) to obtain working solution at eight concentration levels. Calibration standards were prepared by spiking working solutions with drug-free human plasma to the final concentration, which ranged from 0.25-2.50 µg/mL for maraviroc, 0.5-20.0 µg/mL for lopinavir, and 20-2,000 ng/mL for the other ARVs. Quality control (QC) samples were prepared separately in the same way to create low, medium, and high controls at 0.75, 1.20, and 2.20 µg/mL for maraviroc; at 1.50, 8.00, and 18.00 µg/mL for lopinavir; and at 60, 450, and 950 ng/mL for the other ARVs. The IS working solution was prepared by diluting the stock solutions in a mixture of methanol and water (50:50, v/v) to a final concentration of 50 µg/mL. All standard stock solutions, working solutions, and QC samples were stored at -20°C until use.

TABLE 1. Gradient programmed for the mobile phase: Solvent A (Acetonitrile) and Solvent B (0.1% formic acid in water).

Time (min)	Flow (mL/min)	% Solvent A	% Solvent B
0.0	0.45	20	80
1.0	0.45	30	70
3.0	0.45	35	65
4.0	0.45	32	68
9.0	0.45	45	55
12.0	0.45	40	60
14.0	0.45	65	35
16.0	0.45	20	80

Sample Preparations

For solid phase extraction (SPE), OASIS HLB: Hydrophilic-Lipophilic-Balanced reversed-phase sorbent 30 mg 1 mL (Waters Corporation, Milford, MA, USA) was used for sample preparation. SPE HLB cartridges were initially conditioned with 1 mL of methanol and equilibrated with 1 mL of Milli-Q water before use. Twenty µL of IS (50 µg/mL) was added into 1.0 mL of plasma samples. Then, 1 mL of plasma samples were aspirated into the wetted preconditioned SPE HLB cartridges. The plasma components were then washed in two steps with wash solvent, as follows: step 1) 1 mL of ammonia solution and 5% methanol in water (2:98, v/v); and, step 2) 1 mL of ammonia solution and 50% methanol in water (2:98, v/v). ARVs were subsequently eluted from the dried columns using 0.5 mL of an eluting solution (acetic acid and methanol, 2:98, v/v). Three hundred µL of elute was then diluted with 200 mL of 0.1% formic acid before being injected into the UPLC system.

Bioanalytical method validation

The developed method was fully validated according to the Guidance for Industry, Bioanalytical Method Validation by the U.S. Food and Drug Administration (FDA).[24]

1) Selectivity and Sensitivity

Selectivity was examined using six sources of drug-free plasma, for which the result should not have interfering peaks at the retention times of the 11 ARVs and IS. Sensitivity at the lower limit of quantification (LLOQ) was also examined by dilution of standard compounds in plasma, which were extracted and then quantified at the lowest detectable concentration.

2) Intraday and Interday Precision and Accuracy

Intraday and interday precision and accuracy were examined by analysis of six replicates of LLOQ, LQC, MQC, and HQC for three consecutive days. Percentage of relative error (%RE) indicating accuracy and percentage of coefficient of variation (%CV) indicating precision were calculated. Both %RE and %CV should be within ±20% at LLOQ and ±15% at other concentrations.

3) Linearity and Calibration Curve

A calibration curve was represented by a linear regression model, y=mx+b and weighting by 1/x, where y was the ratio of peak area of analyte to the peak area of IS, x was the concentration at different levels, including 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL for MRV; 0.5, 2.0, 5.0, 10.0, 15.0, and 20.0 µg/mL for LPV; and, 20.0, 100.0, 500.0, 1,000.0, 1,500.0, and 2,000 ng/mL for all 9 other ARVs. All calibration ranges yielded linear relationships with coefficient of determination (r^2) that exceeded 0.995.

4) Extraction Efficiency

Extraction efficiency of the method was performed by comparing peak areas of extracted samples at three QC concentrations, with peak areas of non-extracted standard solutions at the same concentrations. Recovery of the analyte was not required to be 100%, but the extent of recovery of an analyte should be precise and reproducible.

5) Stability

Stability of analysis was performed by analyzing three replicates of plasma spiked with three QC concentrations under various conditions with freshly prepared samples. The first condition was freeze and thaw, in which plasma samples were frozen at -20°C and thawed at room temperature for three cycles before analysis. For

short-term stability test, plasma samples were stored at room temperature for 6 hours before analysis. For post-preparative stability test, vials of plasma samples were placed in the auto-sampler at 8°C for 10 hours before analysis. Lastly and for the long-term stability test, plasma samples were frozen at -20°C for 3 months before analysis. The acceptable percentage of variation in each condition must be within ±15%.

6) Matrix Effect

The matrix effect was determined by analysis of six replicates of QC samples at three different concentrations in the blank extracts and in the solution. The matrix factor (MF) was calculated as the ratio of the peak area between presence and absence of matrix. An average matrix value within the range of 0.8-1.2 indicates no matrix effect.

RESULTS

This method demonstrated a good separation result for 11 ARVs in 16 minutes as shown with PDA spectrum in Fig 1. The capacity factor (k′) was in the range of 1.832-34.628 and the resolution factor was in the range of 1.577-39.263 as summarized in Table 2. The specific wavelength selected for each drug was 210 nm for maraviroc and lopinavir; 240 nm for nevirapine, raltegravir, atazanavir, ritonavir, efavirenze, and etravirine; and, 260 nm for dolutegravir, darunavir, and elvitegravir. Wavelength selection was based on the requirement for high sensitivity and specificity. There were no interfering peaks for retention time of 11 ARVs in selectivity testing (Fig 2). The lower limit of quantification (LLOQ) was 0.25 µg/mL for maraviroc, 0.5 µg/mL for lopinavir, and 20 ng/mL for the remaining 9 ARVs. The accuracy and precision of the proposed method were acceptable, as summarized in Table 3. Method accuracies were in the range of 92.23-113.51%. Interday and intraday precision was also observed, with %CV in the range of 1.60-10.43%. This method also showed good linearity, with a coefficient of determination (r^2) greater than 0.996 (Table 4). Although extraction efficiency was in the range of 77.57-101.66%, the recovery of each ARV was consistent, precise, and reproducible with a %CV range of 1.61-9.27 (Table 4). Under the various conditions used for stability testing, the percentage of variation for each condition was within an acceptable range (Table 5). For matrix effect, a matrix factor (MF) between 0.92-1.19 indicated that other substances in the sample cannot significantly affect the accuracy and precision of the method.

DISCUSSION

These were only two previously published studies in ARVs analysis by UPLC-PDA method. Analysis of 8 ARVs took longer than 9.5 min in a study by Antunes, *et al.*[23] and 14 min for 10 ARVs in a study by Elens, *et al.*[22] Although the resolution factors between elvitegravir and lopinavir were lower than 2.0 (1.577), their elution peaks and analytic peaks were also completely separated. The LLOQ is defined as the lowest concentration that can be quantified below the suggested minimum target of trough concentrations in patients with HIV. In our method of detections, 10 ARVs were detected within accuracate time and in therapeutic recommended doses. The exception was Maraviroc, which needed higher concentrations 50 ng/mL for detection by UPLC method. However, these methods can measure peak concentrations when dosed 150 mg or more (recommended dose is 300 mg bid). The lower LLOQ values of ritonavir, nevirapine, atazanavir, and efavirenze indicate higher sensitivity than previously published UPLC-PDA methods.[22-23] As shown in Fig 1, Lopinavir and Maraviroc can absorb only at a wavelength of 210 nm. At 190 -210 nm, the analysis will be disturbed by the other substances' noise. It's better to avoid analysis below 210 nm due to low sensitivity of method. Therefore, the analytical concentration range of Lopinavir and Maraviroc are higher or different from the others. Solid phase extraction was optimized in order to obtain a more rapid and simple procedure for routine analysis than was previously published[19], due to no evaporating step that consumes about 45-60 minutes. Then, our UPLC method may have an opportunity in therapeutic monitoring in HIV patients who are treated with several antiretroviral drugs because it is a cheaper method and can detect broader separated chromatograms. The patients who have low plasma antiretroviral drugs level will result in administration failure and treatment failure. In the opposite side, the patients who have high plasma drugs level will need awareness of more adverse drug effects and toxicities.

CONCLUSION

A novel, sensitive, accurate, and reproducible UPLC/PDA method for quantification of 11 currently prescribed and monitored antiretroviral drug concentrations in human plasma was successfully developed and validated according to USFDA guidelines. The authors propose this method for therapeutic drug monitoring in HIV patients who are treated with several drugs regimen.

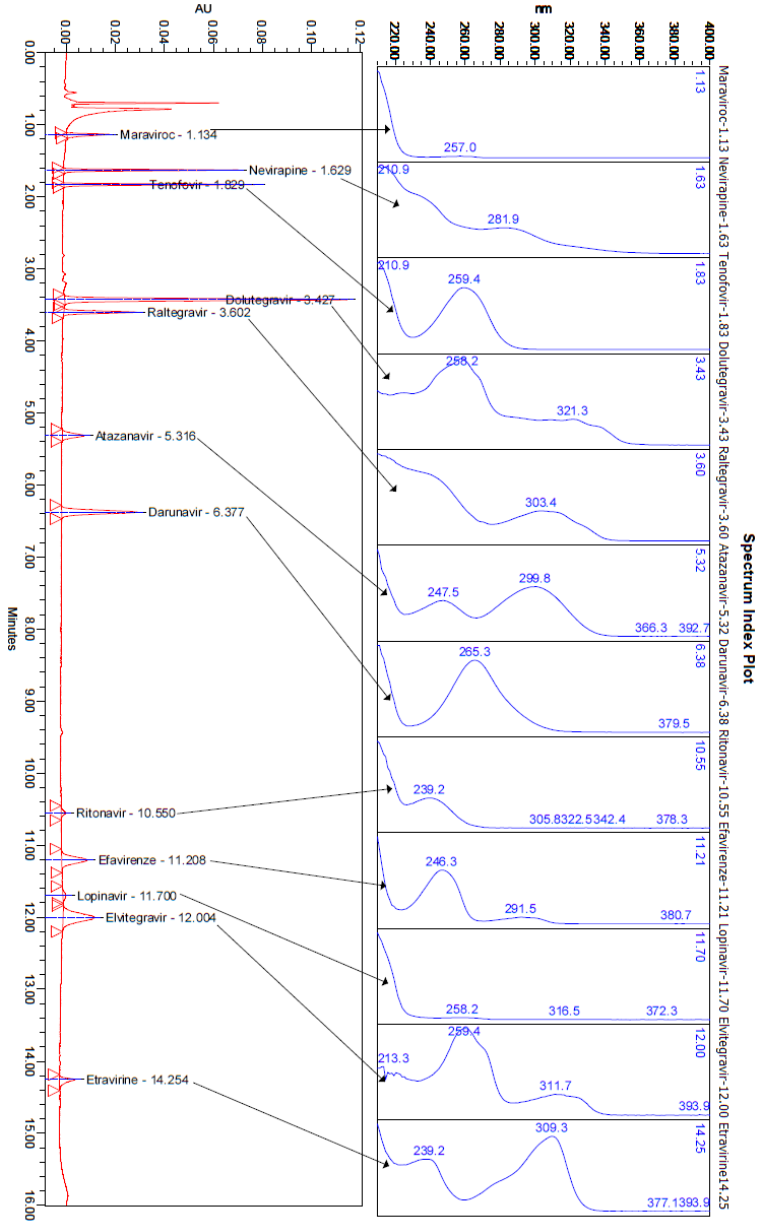


Fig 1. Chromatograms obtained during simultaneous separation of 11 ARVs on the Left panel. PDA Spectrum of each chromatogram has been show on the Right panel, which is the specific pattern of absorbance of each drug.

TABLE 2. Retention time, capacity factor (k′), and chromatogram resolution of ARVs.

Compound	Retention time	Capacity factor	Resolution
Maraviroc	1.134	1.832	-
Nevirapine	1.629	3.070	11.321
Tenofovir	1.829	3.570	6.080
Dolutegravir	3.427	7.566	35.372
Raltegravir	3.602	8.004	2.998
Atazanavir	5.316	12.287	20.173
Darunavir	6.377	14.940	10.309
Efavirenze	10.550	25.371	39.263
Ritonavir	11.208	27.014	4.671
Lopinavir	11.700	28.244	2.942
Elvitagravir	12.004	29.003	1.577
Etravirine	14.254	34.628	14.817

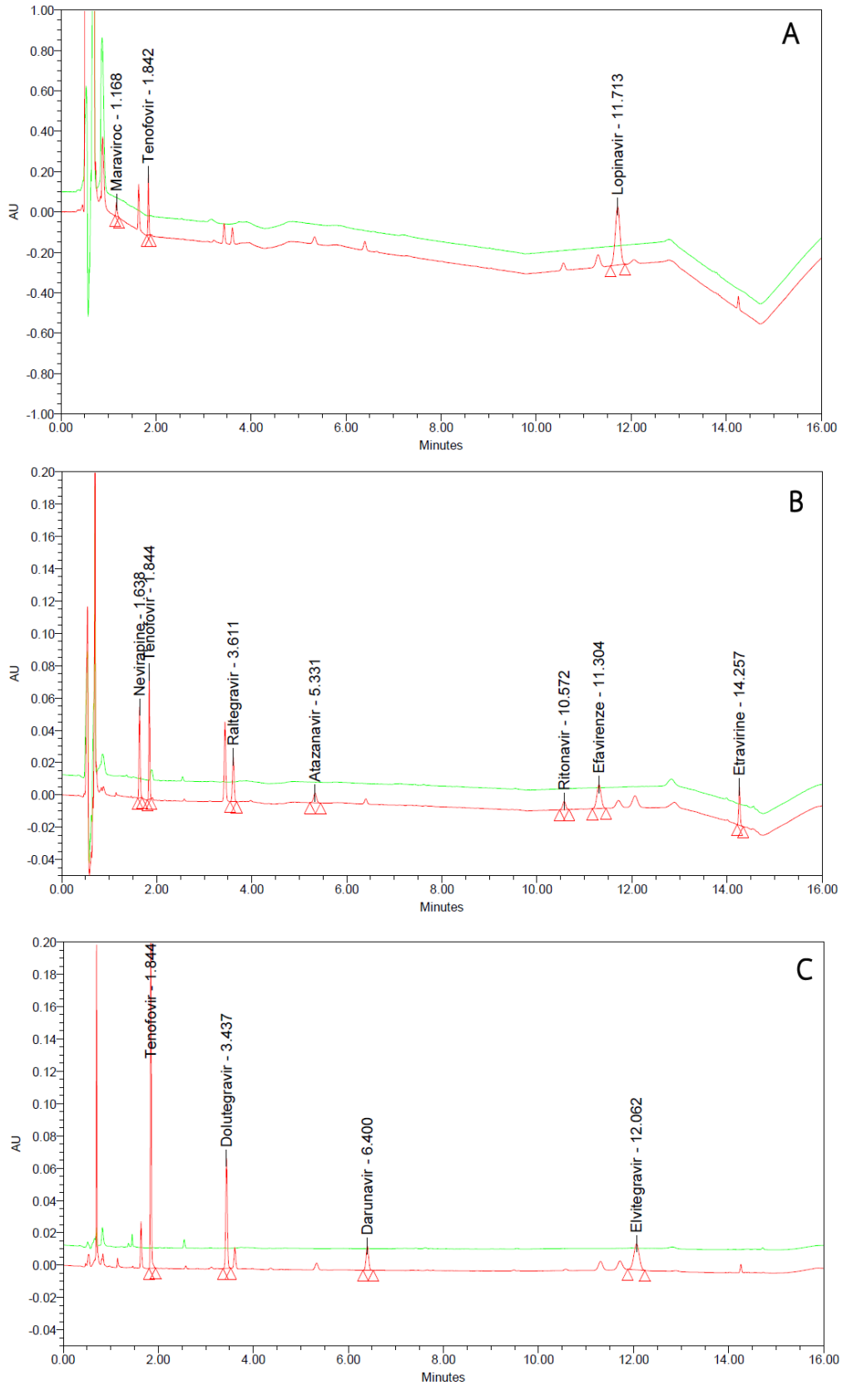


Fig 2. Chromatograms of blank plasma and blank plasma spiked with ARVs: (A) measurement at 210 nm; (B) measurement at 240 nm; and, (C) measurement at 260 nm.

TABLE 3. Accuracy and interday and intraday precision of ARVs

ARVs	LLOQ			LQC			MQC			HQC		
	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)
Maraviroc	99.85	6.90	6.37	96.79	5.04	7.01	96.13	3.76	7.49	100.44	9.73	8.44
Nevirapine	104.11	6.88	5.15	102.43	6.61	7.19	100.44	9.57	7.98	96.24	3.95	3.84
Dolutegravir	102.38	8.20	6.48	94.83	5.08	5.03	96.48	4.82	7.85	103.59	6.80	5.89
Raltegravir	105.47	4.93	5.15	100.44	6.57	7.79	101.73	1.60	9.87	102.68	7.73	9.73
Atazanavir	113.51	6.14	9.49	100.75	7.04	7.68	97.08	5.35	6.66	97.40	3.42	8.30
Darunavir	101.39	2.14	3.26	92.23	5.08	5.89	97.22	4.87	5.22	102.05	2.84	5.89
Efavirenze	98.50	5.49	4.96	95.74	7.06	8.94	103.15	5.15	5.30	108.59	1.77	4.04
Ritonavir	112.98	5.77	7.37	98.57	6.86	6.98	94.76	5.56	6.13	94.91	4.73	5.00
Lopinavir	107.71	7.57	4.96	99.84	8.27	6.35	109.95	2.64	3.06	95.46	8.92	8.38
Elvitegravir	109.15	4.20	4.83	100.61	4.53	9.47	100.43	10.43	7.63	102.20	8.44	7.18
Etravirine	108.39	8.20	5.42	99.71	4.19	7.72	103.15	4.17	8.04	108.75	3.30	5.94

Abbreviations: n = number of replicates; LLOQ = lower limit of quantification; LQC = lower quality control; MQC = mid-quality control; HQC = high quality control

TABLE 4. Concentration range, linearity, recovery of extraction, and target concentrations of ARVs.

ARVs	Concentration range (ng/mL)	Coefficient of determination (r^2) (n=3)	Recovery of extraction (%RE) (n=6)	Target concentration (ng/mL)
Maraviroc	250-2,500	0.996932	89.38	>50
Nevirapine	20-1,000	0.998057	93.39	>3,500
Dolutegravir	20-1,000	0.997449	85.12	>40
Raltegravir	20-1,000	0.997997	83.52	>65
Atazanavir	20-1,000	0.997843	85.13	>200
Darunavir	20-1,000	0.997304	81.89	>3,300
Efavirenze	20-1,000	0.998031	101.66	>1000
Ritonavir	20-1,000	0.998474	95.60	>2,100
Lopinavir	500-20,000	0.996364	77.57	>1,000
Elvitegravir	20-1,000	0.997734	83.47	>200
Etravirine	20-1,000	0.996066	85.28	>300

Abbreviation: n = number of replicates

TABLE 5. Stability of ARVs.

ARVs	Variation (%) (n=3)			
	Freeze and thaw	Short-term	Long-term	Post-preparative
Maraviroc	1.42-9.58	1.46-7.82	2.20-10.77	2.04-5.02
Nevirapine	2.13-5.12	0.39-5.02	4.67-13.32	2.44-4.00
Dolutegravir	2.09-10.22	3.26-9.49	0.41-6.99	1.80-7.75
Raltegravir	1.15-11.92	1.69-10.38	3.66-9.97	0.17-4.75
Atazanavir	2.41-11.89	5.02-11.17	0.80-10.53	0.36-2.03
Darunavir	4.33-12.40	2.10-11.86	3.86-7.81	0.41-3.97
Efavirenze	2.85-9.15	1.31-8.23	2.77-6.86	1.29-5.32
Ritonavir	5.16-10.76	1.11-5.06	0.11-10.29	0.65-1.38
Lopinavir	0.06-13.44	2.32-3.79	0.25-8.34	3.38-8.06
Elvitegravir	1.07-2.38	1.33-6.04	1.86-3.31	0.98-4.00
Etravirine	3.60-14.02	2.06-5.62	2.61-12.09	0.17-6.75

Abbreviation: n = number of replicates

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Analysis of Extrahepatic Collateral Arteries in Transcatheter Arterial Chemoembolization of Hepatocellular Carcinoma

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ABSTRACT

Objective: Development of extrahepatic collateral arteries (ECAs) supply to hepatocellular carcinoma (HCC) can interfere effective control of tumor by transcatheter arterial chemoembolization (TACE) treatment. The aim of this study is to analyze the prevalence and causative factors of ECAs to HCC and complications of TACE through each ECAs.

Methods: We performed a retrospective review of total 1,374 procedures from 639 patients with a diagnosis of HCC who were sent for TACE (range from 1 - 16 sessions, mean = 2.87 sessions) from January 2008 - May 2010. Prevalence, causative factors such as size and location of the tumor and previous treatment were analyzed.

Results: There are 122 (19.09%) from 639 patients that showed ECAs supplying the tumors. The prevalence of ECAs to HCC was 34.39% from right inferior phrenic artery, 30.68% from omental artery, 9.52% from left gastric artery, 5.82% from left inferior phrenic artery, 5.29% from colic branch of superior mesenteric artery (SMA) and 4.23% from intercostal artery. Statistical analysis showed that tumor size of more than 8-10 cm and location at hepatic surface, especially liver bare area were significantly associated with the presence of ECAs ($p<0.01$). The cumulative probability of ECAs formation increases with a number of the sessions of TACE.

Conclusion: Our observation indicates that the factors which could influence ECAs formation included tumor size of more than 8-10 cm and tumor location at surface area, especially liver bare area. As the number of TACE sessions increased, the cumulative probability of the presence of ECAs also increased. TACE through the ECAs is a relatively safe procedure. To recognize and get familiar with the presence of ECAs to HCC are essential to improve treatment outcome of the patients.

Keywords: Extrahepatic collateral arteries; hepatocellular carcinoma (Siriraj Med J 2018;70: 247-253)

INTRODUCTION

Hepatocellular carcinoma is the most common primary malignancy of the liver, with an annual incidence of more than 1 million worldwide.[1]

Surgical resection is the standard treatment for HCC. However, most patients with HCC were inoperable because of advanced stage at time of the presentation and would miss the optimal chance of operation.[2]

Transcatheter arterial chemoembolization (TACE) has been widely used as the effective therapeutic method or as an alternative palliative treatment. TACE is based on the fact that the normal liver receives a dual blood supply from the hepatic artery and the portal vein, but HCC is exclusively supplied by the hepatic artery.[3]

The extrahepatic collateral arteries (ECAs) to the liver are established in various conditions. The development of ECAs in HCC is certainly a situation that limits the efficacy of TACE. Chemoembolization through ECAs

Abbreviations: ECAs = extrahepatic collateral arteries, HCC = hepatocellular carcinoma, TACE = transcatheter arterial chemoembolization

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can be attempted to improve the therapeutic efficacy of TACE.

Previously, there was no study in Siriraj Hospital to analyze the prevalence and other concerns about the development of ECAs to HCC. Therefore, we retrospectively reviewed the prevalence of ECAs supplying HCC on digital subtraction angiography (DSA), causative factor and complication of TACE through each ECA in Siriraj Hospital.

MATERIALS AND METHODS

The study was approved by the Siriraj Institutional Review Board (Si.025/2553(EC1)). Between January 2008-May 2010, we retrospectively reviewed 1374 procedures (ranged from 1 - 16 sessions, mean = 2.87 sessions) from 639 consecutive patients with diagnosed HCC who were sent for TACE in Interventional Radiology (IR) Unit, Department of Radiology, Siriraj Hospital. Of the 639 patients, there were 521 males and 118 females with age ranging from 14 - 87 years (mean = 58.78 years). There were 49 patients who underwent radiofrequency ablation (RFA) before 1st TACE, and 37 patients underwent surgery before 1st TACE.

The sequence of study and intervention

General protocol of the patients who were sent to for TACE in our IR unit included dual phase enhanced CT scan of liver (64-section CT scanner: Lightspeed VCT; GE medical System) before initial TACE session. Then, initial angiogram with TACE was performed. Dual phase enhanced CT scan was done 4-5 weeks after initial TACE to evaluate residual tumor. If indicated, 2nd TACE was performed in the following week. After that, the 2nd dual phase enhanced CT was done to evaluate residual tumor in next 4-5 weeks and repeated TACE was done if indicated.

Before the TACE procedure, the anatomical imaging (CT or MRI of liver) were reviewed and planning for selecting the relevant arterial supply to the HCC mass included direct supply from hepatic artery and possible supply from extrahepatic collateral arteries as correlated with tumor characteristics that have risk of developing extrahepatic collateral arteries such as large tumor size, exophytic, extending to subcapsular areas or previous TACE procedure with hepatic artery dissection, stenosis and occlusion by repeated TACEs or evidence of previous tumor staining. The TACE procedure in IR unit, Siriraj Hospital were performed by chemo infusion using 5-FU 500 mg + Lipiodol 5 ml and Mitomycin-C 20 mg + Lipiodol 5 ml until arterial flow stasis was achieved. If the initial hepatic arterial blockade was insufficient because of a largely sized mass or arterioportal shunting, then embolization was performed with absorbable gelatin sponge particles (about 1 mm in diameter; Gelfoam; Upjohn, Kalamazoo, MI) soaked in 10 mL of nonionic contrast medium.

Statistical analysis

The angiogram and dual phase CT scan were retrospectively reviewed by an interventional radiologist who had experience in IR field for more than ten years and a 3rd-year radiology resident. Fisher's exact test for nominal variables and t-test for continuous variables were used and P values < 0.05 were considered to indicate statistical significance. SPSS statistical analysis software (SPSS Inc.) was used.

RESULTS

The mean patient's age was 58.78 years (range from 14 - 87 years) included 521 males and 118 females. There was no significant association of the patient's age and gender with the formation of ECAs.

In 122 patients (19.09%) from 639 patients, 189 ECAs were found to supply HCC. The number of ECAs per patient were one (71 patients), two (36 patients), three (11 patients), four (2 patients) and five ECAs (2 patients). The mean number of ECAs was 1.54 ECAs per patient. The prevalence of each ECA was shown in Table 1, Figs 1-4.

The tumor size and location were shown in table 2. The overall mean tumor size was 8.8 cm. The mean tumor size of first TACE was 9.6 cm. The mean tumor size of repeated TACE was 8 cm.

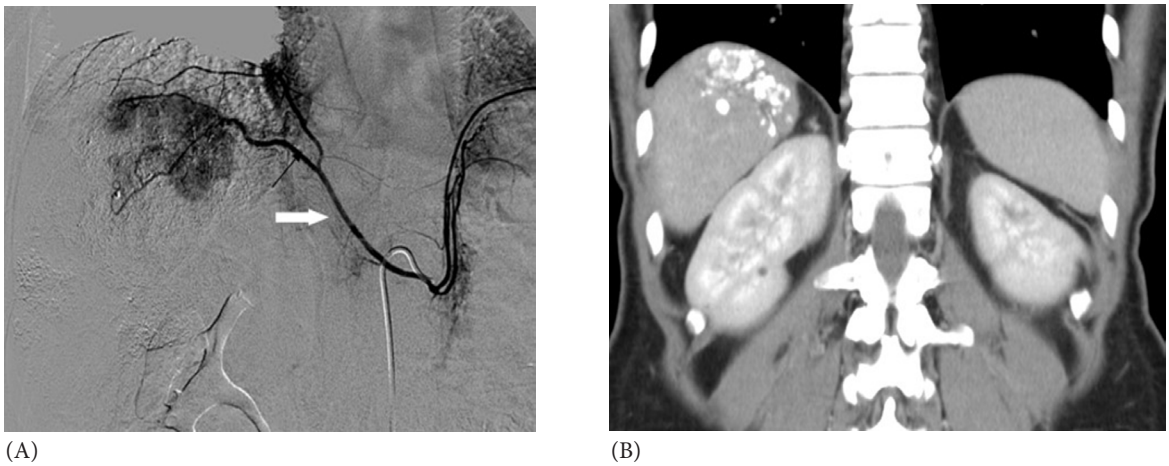
Univariate analysis showed that tumor size ($p < 0.01$), and location ($p < 0.01$) especially liver bare area were significantly associated with the formation of ECAs (Table 3). The cumulative probability curve (Table 4) showed that, as the number of TACE sessions increased, the probability of ECAs formation also increased.

There were 48 patients that underwent previous radiofrequency ablation (RFA) before first TACE, and all of these patients showed no ECA formation to supply HCC. There were 37 patients that underwent previous surgical resection before first TACE and 9 (24.32%) patients showed the formation of ECAs (P value = 0.536).

From 189 ECAs in 122 patients, embolization was done in 101 ECAs from 78 patients (Right inferior phrenic artery (RIPA) = 43, gastroduodenal artery (GDA) = 26, right inferior adrenal artery (RIAA) = 3, intercostal artery (ICA) = 6, left gastric artery (LGA) = 10, left inferior phrenic artery (LIPA) = 4, right renal capsular artery (RRCA) = 5, cystic artery = 1, colic branch from

TABLE 1. Prevalence of extrahepatic collateral arteries (ECAs).

Origination of ECAs	Incidence of ECAs	Percentage of ECA present (%)
Right inferior phrenic a.	65	34.39
Omental a.	58	30.68
Left gastric a.	18	9.52
Left inferior phrenic a.	11	5.82
Colic branch from SMA	10	5.29
Intercostal a.	8	4.23
Right renal capsular a.	6	3.17
Cystic a.	5	2.64
Right inferior adrenal a.	4	2.11
Right gastric a.	2	1.05
Right middle adrenal a.	1	0.52
Right superior adrenal a.	1	0.52
Total	189	100



(A) (B)

Fig 1. (A) A 50-year-old man with hepatocellular carcinoma was sent for 5th TACE. The right inferior phrenic angiogram show abnormal tumor staining at superior part of right lobe of liver supplied by dilated right inferior phrenic artery (arrow), (B) CT liver show HCC mass at right hepatic dome with partial lipiodol staining.

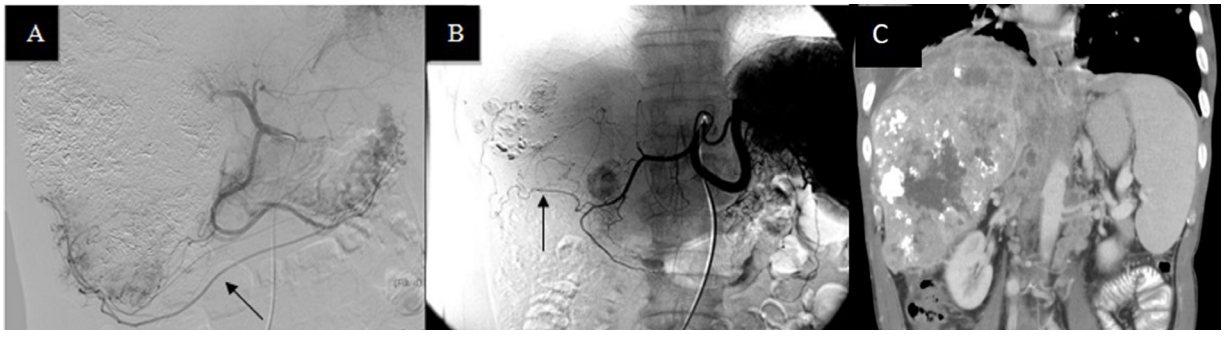


Fig 2. (A) A 40-year-old man with hepatocellular carcinoma was sent for 1st TACE. A. Gastroduodenal artery angiogram shows tumor staining at inferior part of right lobe of liver supplied by omental branch of right gastroepiploic artery (arrow). (B) TACE was performed in this ECA and showed increased lipiodol staining and decreased tumor vascularization (arrow). C. CT liver shows a large HCC at right lobe of liver with partial lipiodol staining.

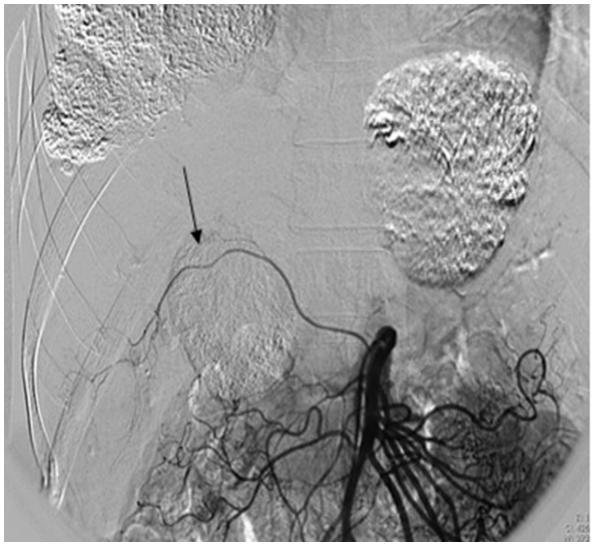
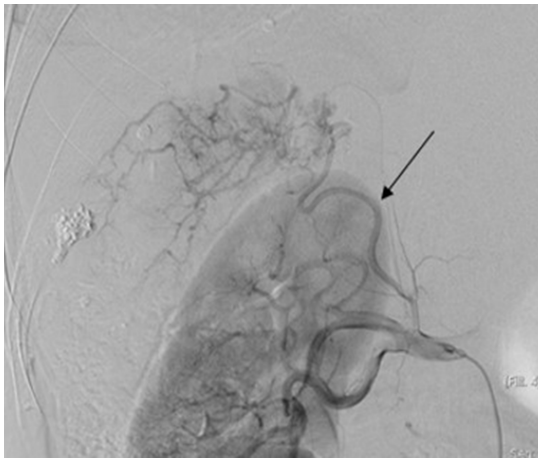


Fig 3. The same patient in fig 2 showed abnormal tumor staining at inferior part of right lobe of liver supplied by colic branch of superior mesenteric artery (arrow).



(A)



(B)

Fig 4. (A) A 50-year-old man with hepatocellular carcinoma with the right renal angiogram revealed abnormal tumor staining at inferior part of right lobe of liver supplied by right renal capsular artery (arrow) which has common origin to right inferior adrenal artery. (B) CT liver shows HCC mass at inferior part of right lobe of liver near the upper pole of right kidney.

SMA (superior mesenteric artery) = 2 and right middle adrenal artery (RMAA) = 1).

The complication occurred were described as follows, myelopathy or spinal cord infarction in 1 / 6 patients from embolization of ICA, which probably resulted from the inadvertent embolization of spinal branches arising from intercostal artery or non-target embolization; fever, abdominal pain and nausea/vomiting in 41 patients from RIPA (24 / 43 patients), ICA (1 / 4 patient), RIAA (1 / 3 patient), GDA (12 / 26 patients), LGA (3 / 10 patients); back pain from RIPA (2 / 43 patients) and hiccough from RIPA (1 / 43 patient). To prevent some serious complications especially myelopathy or spinal cord infarction, a good planning before TACE, including evaluation for a shunt and choice of the vessels used for the Lipiodol infusion prior to TACE, are very important to achieve a good overall result.

DISCUSSION

The development of ECAs in HCC is certainly a situation that limits the efficacy of TACE. There were several studies that reported the prevalence of ECAs and their concern. Wang YL et al.,[2] reported that extrahepatic blood supply tumors could be found in 43.1% patients with unresectable HCC. Okazaki et al.[4] found that one or more focuses of recurrence of HCC after hepatectomy in 38.2% patients (26/68) were fed not only by hepatic arteries but also by ECAs. Jin Wook Chung et al.,[3] showed that 82 (17%) of 479 patients showed 108 ECAs supplying tumor at initial presentation and 70 (14%) during repeated TACE sessions. They also found that the tumor size was a significant causative factor for development of these collateral vessels. Although they were differences among reported studies about incidence of ECAs, they had influenced the efficacy of chemoembolization of unresectable HCC. The prevalence and other concerns about ECAs formation have not been previously evaluated in Siriraj Hospital.

In our study, the tumor size was an important factor associated with ECAs formation (P value < 0.01) and the tumor size of > 8-10 cm, had the highest prevalence of ECAs. The larger size of tumor also have tendency to find ECAs formation as shown in Table 4., which correspond to the study of Wang YL et al.,[2] and Jin Wook Chung et al.[3] A surface tumor location, especially bare area (P value < 0.001) is a prerequisite for formation of ECAs which corresponds to the study from Jin Wook Chung et al.[3] As the number of TACE sessions increased, the cumulative probability of the presence of ECAs was also increased, corresponding with the previous study from Wang YL et al.,[2] and Jin Wook Chung et al.[3]

TABLE 2. Tumor size and correlation with presentation of extrahepatic collateral arteries (ECAs).

Tumor size	ECAs		Percentage of		P value
	absent	present	ECA present (%)	Total	
<2 cm	87	0	0	87	
2-4 cm	177	23	11.5	200	
4-6 cm	86	27	23.89	113	
6-8 cm	49	14	22.22	63	<0.01
8-10 cm	41	27	39.71	68	
>10 cm	77	31	28.7	108	
Total	517	122	19.09	639	

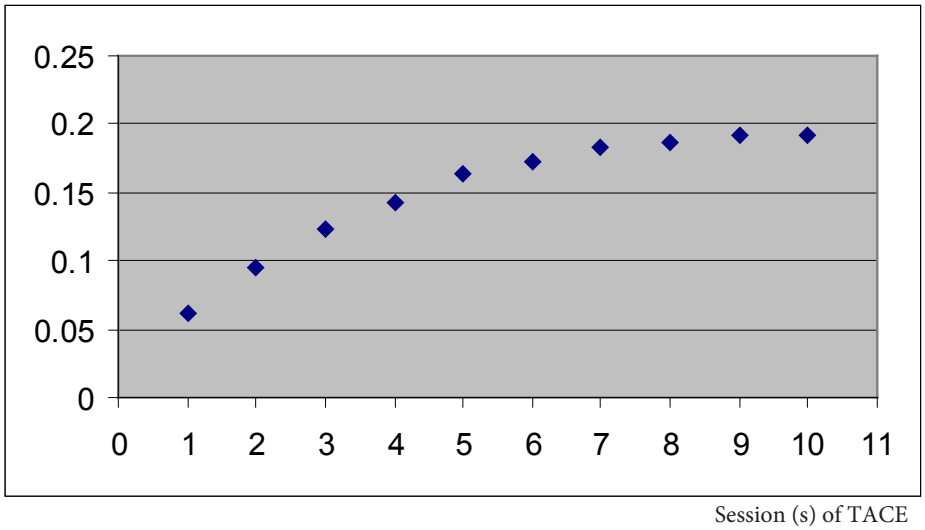
TABLE 3. Tumor size and correlation with presentation of extrahepatic collateral arteries (ECAs)

Location[1]		ECAs		Persentage of		P value
		Absent	Present	ECA present (%)	Total	
Surface	Bare area[2]	34	41	54.66	75	<0.01
	non-bare area	253	69	20.11	322	
Non-surface		230	12	5.06	242	<0.01
Total		517	122	19.09	639	

[1] If a tumor showed exophytic growth or it was observed to reach the liver margin on CT, then it was considered to have a surface location, but if it did not reach the liver margin, then it was considered to have a nonsurface location.[3]

[2] Posterior surface hepatic segment 7 and posterior half of the diaphragmatic surface of hepatic segment 8.[3]

TABLE 4. Cummulative probability curve of extrahepatic collateral artery formation of hepatocellar carcinoma.



Many articles have reported the prevalence of each ECAs. Shiro miyayama et al.,[5] reported incidence of RIPA (83%), cystic a. (24%) and omental a. (13%). Jin Wook Chung et al.,[3] reported incidence of RIPA (49.8%), omental a. (15.6%) and cystic a. (8.8%); Wang YL et al.,[2] showed incidence of RIPA (38.5%), SMA (23.1%) and LGA (12.8%). All mentioned studies showed that the right inferior phrenic artery was the most common ECA, corresponding to our result, (RIPA 34.3%, omental 30.6% and LGA 9.5%). The large tumor size which is located in contact with diaphragm especially bare areas is likely to be supplied from inferior phrenic artery. The tumor is probably supplied by omental branch if it located near surface of right hepatic lobe and medial segment. However, this omental branch can also supply a tumor at any location of liver due to it traverses along the greater omentum which is relatively mobile.

The procedure that causes ECAs in the literature included hepatic a. occlusion by surgical ligation, hepatic arterial interruption by repeated TACE or arterial dissection, previous abdominal operation due to postoperative omental or peritoneal adhesion and recurrent tumor at the resection margin.[6] However, ECAs supplying HCC also develop in the anatomical location of HCC, although the hepatic arterial supply remains intact.[7,8] In our study, 38 procedures from 10 patients who underwent previous surgical resection before 1st TACE developed ECAs. However, there was no significant correlation between previous treatment and development of ECAs (due to P-value=0.536 (>0.05)). We suggest that more patients who had previous surgical resection should be further evaluated for this correlation.

The patients who underwent previous RFA before TACE showed no ECA formation. Our study showed that RFA has no correlation with ECA formation, probably due to relatively small tumor size for sending to perform RFA.

Complications from chemoembolization of ECAs included cutaneous problems itching, erythema and necrosis from internal mammary, intercostal, or lumbar artery; gastrointestinal erosion, ulceration, or perforation from gastric, omental, and colic branch; paraplegia from spinal branches arising from intercostal or lumbar collateral vessels; cholecystitis or gallbladder infarction from cystic artery; shoulder pain, pleural effusion or basal atelectasis from inferior phrenic artery.[6,8-11]

The complications, which occurred in this study, included myelopathy from 1/6 patients from ICA; fever and abdominal pain with nausea/vomiting in 41 patients (24/ 43 patients from RIPA, 1/ 4 patients from ICA, 1/3 patients from RIAA, 12/26 patients from GDA, 3/10 patients from LGA; back pain 2/43 from RIPA; hiccough 1/43 patients from RIPA. The prevalence of complications from TACE through the ECAs are relatively low. Therefore, TACE through the ECAs are the relatively safe procedure.

Limitation of this study is due to retrospective analysis. We usually look for the ECAs in the patients who are suspected to have another tumor blood supply further than the hepatic artery. However, we could miss unsuspected ECAs in some patients due to many factors such as non-recognition from the interventional radiologists, or the ECAs are too small to be detected. Therefore, the prevalence of ECAs could be underestimated.

Another limitation was another treatment concerned in individual patients. The causative factors that we evaluated were only RFA and previous surgical resection before 1st TACE due to many patients who were treated by RFA and surgical resection were consulted for TACE. However, some patients had RFA or surgical resection between sessions of TACE and could be unpredictable whether this treatment would effect the formation of ECAs or not.

CONCLUSION

Our observation indicates that the factors which could influence ECAs formation include tumor size and tumor location, especially liver bare area (P value<0.01). As the number of TACE sessions increase, the cumulative probability of ECAs formation also increases. To recognize and get familiar with ECAs supplying HCC are essential to improve treatment outcome of HCC and avoid complication of TACE.

Conflict of Interest: None

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Metastatic Malignant Melanoma of the Urinary Bladder: A Case Report and Review of Literature

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ABSTRACT

Objective: Metastatic malignant melanoma of the urinary bladder is extremely rare in clinical practice, herein, we review literature to demonstrate epidemiology, management and prognosis of this rare condition.

Case presentation: A 57-year-old male with history of malignant melanoma of left big toe was referred to the urology division with the complaint of intermittent painless gross hematuria. Cystoscopy revealed multiple bladder masses, hence transurethral resection of bladder tumor was performed. Histologic and immunohistochemical examination revealed metastatic malignant melanoma involving urinary bladder mucosa.

Conclusion: Suspicion of metastasis should be raised in a patient with urinary symptom, especially if the history of malignant melanoma is present. Cystoscopy and biopsy is recommended if metastatic disease is suspected. While long-term survival is poor, management should be individualized according to the patient's conditions, symptoms and severity of disease.

Keywords: Malignant melanoma; metastasis; urinary bladder (Siriraj Med J 2018;70: 254-259)

INTRODUCTION

Metastatic malignant melanoma of the urinary bladder is extremely rare in clinical practice with just thirty two confirmed cases reported in English-language literature. However, a study in autopsy series by Dasgupta et al., showed that 18% of patients with melanoma had bladder metastasis.[1] This indicates that the incidence is likely higher than previous thought and may be due to the fact that many patients are asymptomatic. Due to unusual variety in clinical presentation, its diagnosis may be difficult for most urologists. Besides, this metastatic type shows worse prognosis. This poses significant challenges in diagnosis and management. We report a case of metastatic malignant melanoma to the bladder that presented as intermittent gross hematuria. We also review literature of PubMed with a focus on metastatic malignant melanoma to the bladder. To our knowledge, there were a small number of reported cases in Asian population and this is the first reported case in Thailand.

CASE PRESENTATION

A 57-year-old man previously diagnosed with malignant melanoma of left big toe five months ago, had undergone wide local excision and left groin node dissection. The pathologic findings revealed melanoma with 8-mm tumor thickness, Clark's level IV, negative margin and nodal metastasis. He presented with intermittent, painless gross hematuria for 2 months. Computed tomography of the brain, chest and abdomen revealed multiple pulmonary nodules and a 3.2 cm space occupied by lesion/mass at the posterior wall of urinary bladder.

He subsequently underwent cystoscopy, revealing multiple dark blue/black-pigmented solid nodular lesions on the posterior wall, and was treated with complete

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transurethral resection (Fig 1). Pathological examination confirmed it to be malignant melanoma (Fig 2). As a result, diagnosis of stage IV malignant melanoma of left big toe with metastasis to urinary bladder was made. Due to the multi-organ distant metastatic condition of the patient, the multidisciplinary team consensus was palliative chemotherapy. He received 4 cycles of carboplatin and paclitaxel every three weeks. After the fourth cycle, the disease was stable. However, the patient was lost to follow up eight months post diagnosis.

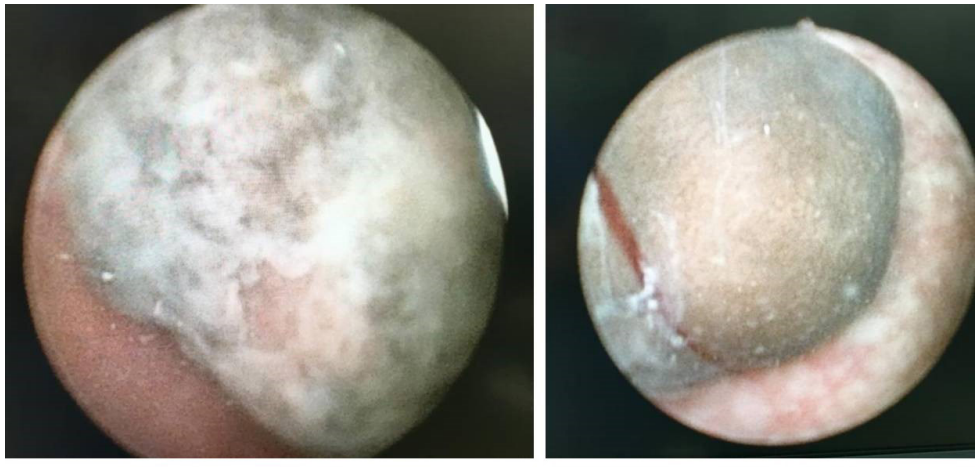


Fig 1. Cystoscopy revealed a large nodular mass (3 cm) with brown pigmentation at right posterolateral wall of bladder (A) and satellite lesion at posterior wall (B).

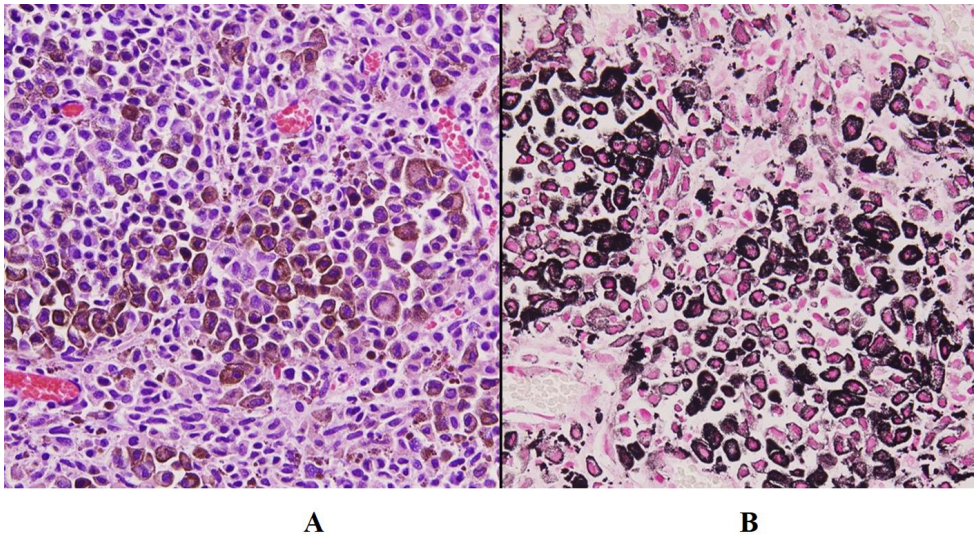


Fig 2. (A) H&E section shows pleomorphic cells with granular eosinophilic cytoplasmand hyperchromatic nuclei. Many cells contain dark brown pigments. (x40) (B) Masson-Fontana stainhighlights melanin pigments in black color. (x40)

DISCUSSION

Metastatic tumors to the urinary bladder are rare; with accounts only about 2 %.[2] They are often categorized as direct extension of tumor from surrounding organs, distant metastasis, and infiltration by lymphoma/leukemia. The most common primary sites of metastasis are prostate, colorectum and cervix. Breast, lung, and skin primaries are less common sources. Primary malignant melanoma is known to occur very rarely in the urinary tract, especially in the bladder. However, metastases of malignant melanoma to the genitourinary tract are relatively common. Therefore, it is very important to distinguish primary from secondary melanomas of the bladder. No evidence of other melanomas and presence of atypical melanocytes at the bladder margin are diagnostic criteria for primary bladder tumor. Metastatic malignant melanoma of the bladder is a rare occurrence in clinical practice with only thirty two other cases of this diagnosis which have been reported in the English literature (Table 1). However, the autopsy series of metastatic melanoma

TABLE 1. Review of English-language literature of metastatic melanoma to the bladder.

Authors	Year	Age	Sex	Primary site	Presenting symptom	Synchronous metastasis	Treatment	Survival
Amar[6]	1964	33	M	Neck	Hematuria	Maxillary lesions	Partial cystectomy	NR
Bartone[7]	1964	70	F	Thumb	Hematuria	Lymph nodes	Partial cystectomy	Died within 2 months
Weston and Smith[8]	1964	69	M	Eyelid	Urinary retention	Widespread	None	Died 5 days after diagnosis
Dasgupta[9] (2 patients)	1965	35	M	Unknown	Hematuria	Axillary lesion	Transurethral fulguration	Died within 4 months
		35	F	Unknown	Hematuria	Inguinal nodes	Tumor cryotherapy and partial cystectomy	NR
Meyer[10] (3 patients)	1974	69	M	Unknown	Incidental	Widespread	Chemotherapy	NR
		60	F	Arm	Hematuria	Unknown	Partial cystectomy	Died 16 month after resection
		42	M	Upper Back	Incidental	Lungs	TUR	Died 2 months after resection
Silverstein et al[11]	1974	56	M	Unknown	Hematuria	Lymph nodes	Intratumor injection of BCG vaccine followed by partial cystectomy	Alive at 8 months follow-up
Tolley et al[12]	1975	48	F	Vulva	Hematuria	Absent	Radical cystectomy	NR
Chin et al,[13]	1982	70	F	Unknown	Hematuria, voiding symptoms, suprapubic pain, fatigue	Bowel	Partial cystectomy Colectomy	NR
Stein and Kendall[3]	1984	50	M	Arm	Hematuria	Absent	TUR, chemotherapy	Alive at 2 year follow-up
Arapantoni-Dadioti et al[14]	1995	28	F	Unknown	Dysuria	Brain, Skin, Lungs lymph nodes	Incomplete TUR	2 months after resection
Ergen et al[15]	1995	65	M	Unknown	Hematuria, Flank pain	Renal pelvis, Stomach	Biopsy	Died within 1 week
Demirkesen et al[16]	2000	45	F	Heel	Hematuria, urinary frequency	Widespread	TUR, chemotherapy	NR
Lee et al[17]	2003	46	M	Back	Hematuria	Widespread	TUR and high-dose IL-2 immunotherapy	NR

Abbreviations: TUR= Transurethral resection; SBRT= Stereotactic body radiotherapy; NR= Not reported

TABLE 1. Review of English-language literature of metastatic melanoma to the bladder.

Authors	Year	Age	Sex	Primary site	Presenting symptom	Synchronous metastasis	Treatment	Survival
Fink et al[18]	2003	38	M	Upper leg	Hematuria	Tonsil Lymph nodes Esophagus Stomach, skin	Embolization of nutrient vessels	NR
Martinez-Giron[19]	2008	49	M	Back	Hematuria	NR	NR	NR
Efesoy and cayan[20]	2011	60	F	Middle finger	Hematuria, weight loss	Lungs	TUR	Died 7 months after resection
Nair et al[21]	2011	54	M	Conjunctiva	Hematuria	Widespread including ureteral and renal pelvic tumors	TUR and laser ablation of ureteral and renal pelvic tumors, Chemotherapy	Died within 3 months of TURBT
Charfi et al[22]	2012	54	M	Esophagus	Unknown	Lymph nodes	TUR	Died within 1 month
Paterson et al[23]	2012	84	M	Upper back	Incidental	No other metastatic lesions	None	NR
Wisenbaugh et al[24] (4 patients)	2012	81	M	shoulder	Hematuria and voiding	Solidary	TUR	Alive at 10 months
		84	M	Skin	symptoms	Small bowel	TUR	Died at 4 months
		85	M	Shoulder	Incidental	Widespread	TUR	Died within 4 months
		62	F	Eye	Hematuria	Lymph node,	TUR	Died within 5 months
					Incidental	small bowel, widespread		
Rishi et al[25]	2014	61	F	Back	Hematuria, dysuria	Brain Lung Skin	TUR Brain radiotherapy High-dose interferon Temozolomide	Died with 2 months
Meunier et al[26]	2015	55	F	Thigh	Hematuria	Liver Spleen Lung	Checkpoint inhibitor	Died
Topal et al[27]	2016	70	F	Eye	Hematuria	NR	TUR,Chemotherapy	Alive at 8 months
Shukla et al[28]	2016	60	M	Thigh	Hematuria	Widespread	TUR	Alive at 1 year follow-up
Krishnan[29]	2017	83	M	Back	Dysuria	Liver, Lymph node, wide spread	Biopsy	NR
Patil[30]	2017	49	F	Flank	Hematuria	Brain	Brain SBRT, checkpoint inhibitors	-
Current report	2017	57	M	Big toe	Hematuria	Lung Lymph nodes	TUR,Chemotherapy	Alive at 8 months

Abbreviations: TUR= Transurethral resection; SBRT= Stereotactic body radiotherapy; NR= Not reported

patients demonstrated an 18%-37% rate of metastatic disease to the bladder, indicating that many patients with this condition remain undiagnosed.[1,3] In addition, the incidence of melanoma has increased faster than any other cancers and accounts for 4% of all newly diagnosed cancers, according to cancer register in the United States.[4] These data suggest that this condition should receive more concern.

The genitourinary tract is a common metastatic site of melanoma. However, isolated metastatic disease to the bladder is relatively rare. The majority of cases are asymptomatic and the most common presenting symptom is hematuria. Other symptoms can mimic infection and benign prostate hyperplasia. When the patient presents with urinary tract symptoms and has a clinical history of previous malignant melanoma, appropriate workup is warranted. As in our patient, the urinary bladder lesions were incidental detected by computed tomography two months after the clinical diagnosis of hematuria. It is important to consider less common causes, such as malignancy in this scenario. The diagnosis of metastatic melanoma to the bladder requires cystoscopy and biopsy. Histopathologic examination should reveal atypical melanocytic cells morphologically similar to the primary tumor. In some cases, like this one, the tumor cells contain melanin pigments; therefore, only compatible morphology and Masson-Fontana positive brown pigments are sufficient to make a diagnosis. However, in cases of amelanotic melanoma, melanoma markers such as S100, HMB45 and melan-A immunostains, are beneficial adjunctive tools.

Several treatments have been proposed to deal with metastatic malignant melanoma including TUR, partial or radical cystectomy, chemotherapy and radiation. Once metastasis occurs, treatment options are limited and survival is generally poor, reflecting a lack of effective systemic therapy. The question for urologists is "Does surgery play a role in the management? If so, should management be transurethral resection (TUR) or cystectomy?" Similar to genitourinary malignancy, metastasectomy for malignant melanoma is controversial, but retrospective data suggested that metastasectomy may be associated with improved overall survival compared to systemic therapy alone.[5]

The choice of surgical strategy should be based on overall prognosis, the patient's health status, presence of local symptoms attributable to bladder metastases, invasiveness of approach, and quality of life factors. Bladder metastasis results in hematuria and voiding symptoms that can sometimes be managed by TUR, although TUR may not be sufficient to remove the entire tumor in many cases. Partial or radical cystectomy seems to be the treatment of choice in potentially curable patients with solitary metastasis. Radical cystectomy is theoretically the first priority of the treatment that would achieve a good tumor control. However if patient has only a single metastatic bladder lesion and denied total cystectomy, partial cystectomy could be recommended with informed risk of tumor recurrence. Up to date, patients have survived less than three years in literature, indicating the aggressive natural history and ominous prognosis of this disease. However, recent literatures have reported that patients were alive at 8-12 months of follow up, even though they had vital organ metastases and underwent TUR for urinary bladder lesion. This surgical treatment was similar to the previous reports, therefore improving systemic chemotherapy trends to be a major role for longer survival. Our patient had concomitant multiple lung metastases and hematuria which was first managed by Transurethral resection of bladder tumor (TURBT). Unfortunately, after 4 cycles of systemic chemotherapy, the patient refused further treatment and was lost to follow up.

CONCLUSION

Reported cases of metastatic melanoma to the bladder are rare. The majority of cases are reported in western countries and also in Japan. To our knowledge, this is the first reported case in the Southeast Asian population. Our case demonstrates the importance of considering a rare diagnosis in a patient with urinary symptom, especially if the history of malignant melanoma is present. Cystoscopy and biopsy are recommended if metastatic disease is suspected. While long-term survival is poor, management should be individualized according to the patient's conditions, symptoms and severity of disease.

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Unusual Pediatric Abdominal Cysts: A Pictorial Review of Imaging Findings

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ABSTRACT

This article presents images and imaging features of unusual abdominal cysts in pediatric population. Recognition of imaging features and their location are helpful in diagnosis and therapeutic decision. Meconium pseudocyst usually has calcified wall and may contain debri or air. Lymphangioma can be located in mesentery or omentum and presents as uni or multilocular cyst. Pseudocyst is the most common complication of acute pancreatitis. Ovarian cyst is sometimes present as an abdominal mass in the newborn and young child. Cystic mass containing fat and calcifications is the pathognomonic finding of mature cystic teratoma. Duplication cyst has gut signature or double wall sign on ultrasound. Communication with bile duct is the helpful clue in diagnosis of choledochal cyst. When adrenal hemorrhage liquefies, it becomes cystic and gradually decreases in size.

Keywords: Cyst; pediatric; abdominal; imaging (Siriraj Med J 2018;70: 260-264)

INTRODUCTION

Abdominal cyst is common problem in infants and children who are referred to the radiology department. Hydronephrosis and cystic renal diseases are the most frequent. Nevertheless, there are many groups of nonrenal cysts which are less common but challenging in clinical practice. A cyst can be palpated either by the parents or a physician, but the child sometimes experiences no symptom. When a cyst is large, the patient usually presents with symptom related with the mass effect such as increasing abdominal girth, abdominal pain, gut obstruction and jaundice.

Plain abdominal radiograph should be the first imaging procedure in every child who presents with abdominal mass. Ultrasound is the modality of choice to evaluate cystic mass while computed tomography (CT) and magnetic resonance imaging (MRI) are helpful in diagnosis and determination of the origin and extension. Although all of them are cystic in appearance, clinical history and imaging findings can provide clues in diagnosis.

This article presents unusual abdominal cysts in pediatric population including meconium pseudocyst, omental and mesenteric cysts, pancreatic pseudocyst, ovarian cyst, cystic teratoma, duplication cyst, choledochal cyst and adrenal hemorrhage.

Meconium pseudocyst

Meconium pseudocyst is a complication of meconium peritonitis secondary to perforation of bowel in utero.[1] Extruded meconium into the peritoneal cavity results in cystic mass formation to seal off the perforation. Calcium deposits are the pathognomonic finding of the meconium pseudocyst which can demonstrate on plain abdominal radiograph and ultrasound. (Fig 1) Occasionally, a scrotal mass is present because the calcifications extend to the scrotal sac. Sonographically, it has a thick, well-defined, echogenic wall and may contain debris or air.[2] Other imaging findings include dilated bowel loops, extraluminal air and intraperitoneal calcifications. There is a wide spectrum of clinical outcomes, ranging from a healthy newborn to a newborn with intestinal obstruction and perforation requiring surgical intervention.[3] Early detection in utero does not correlate with poor postnatal outcome.[4]

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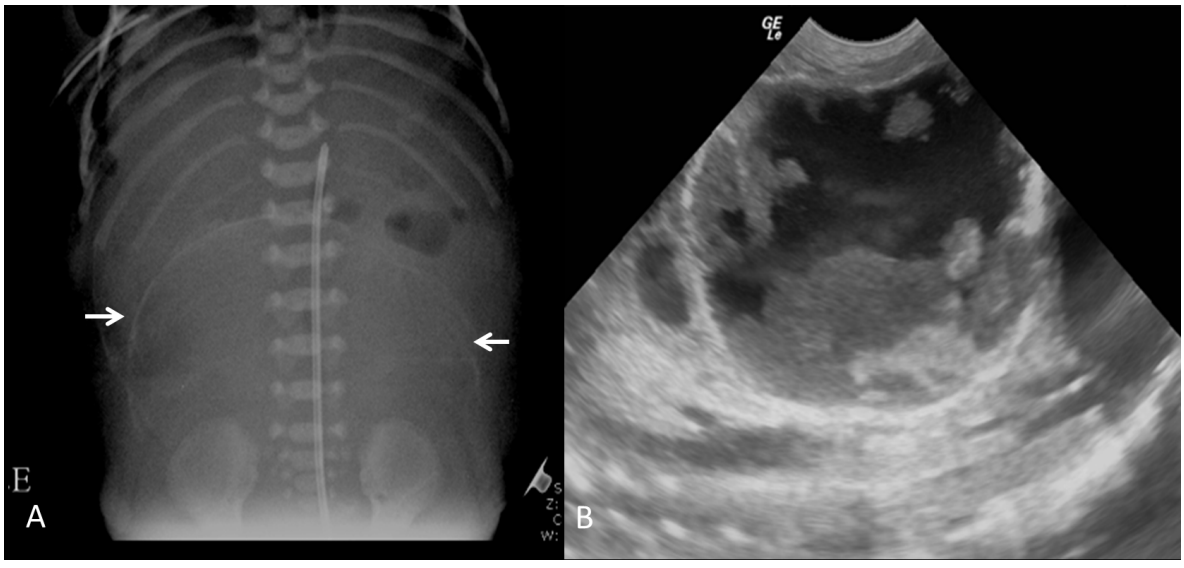


Fig 1. Meconium pseudocyst in a 1-day-old infant with abdominal mass. (A) Abdominal radiograph reveals a large rim calcified mass within the abdomen (arrow). (B) Ultrasound reveals a complex cystic mass.

Omental and mesenteric cysts (Lymphangioma)

It is believed that both conditions are obstructive malformation of the lymphatic vessels. The omental cysts are located in the greater and lesser omentum whereas the mesenteric cysts are located between the leaves of small bowel mesentery and the transverse mesocolon. They can manifest as a unilocular cyst, multilocular cyst or diffuse lymphagiomatosis.[5] Calcifications are rare. Sonographically, lymphangiomas are most often mutilocular cystic masses which are anechoic or contain echogenic debri. Dueto has so thin wall, that it may be seen to fill the entire abdomen, and resembles ascites. Contrast enhanced CT may demonstrate enhancement of cyst wall and septa.[6] (Fig 2) The definitive treatment is surgical excision.

Pancreatic pseudocyst

The incidence of acute pancreatitis has been rising in pediatric population over the past decades.[7] In a series of 589 patients, no etiology was identified in nearly 25%

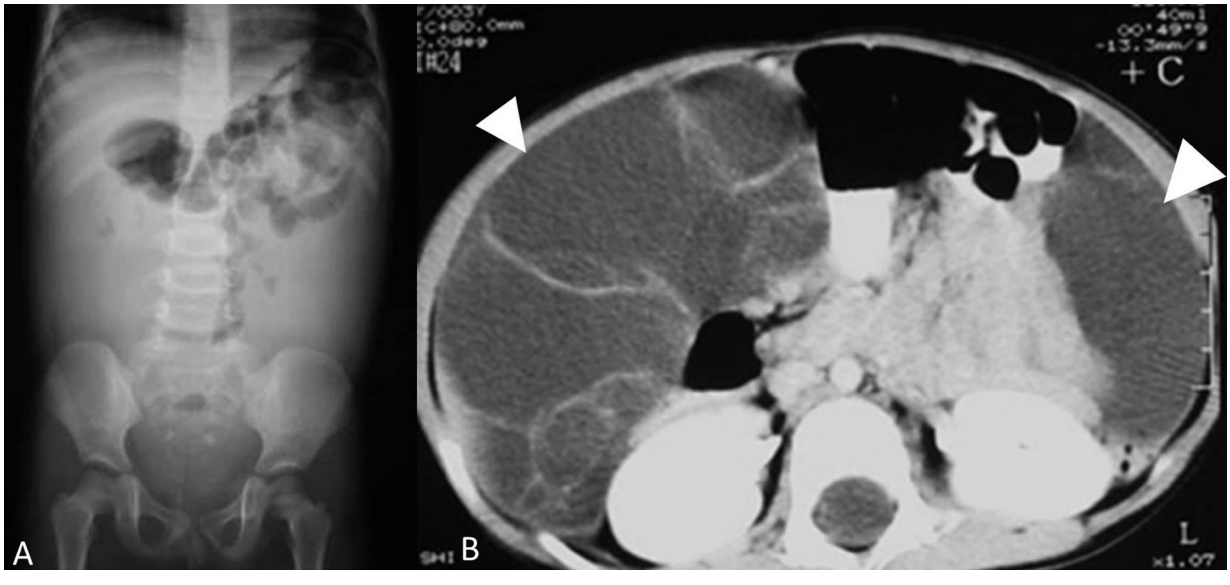


Fig 2. Omental cyst in a 3-year-old girl with chronic abdominal pain. (A) Mass effect within abdomen causes displacement of bowel loops on AP view of plain abdominal radiograph. (B) Axial CT demonstrates the cystic mass arising from the omentum (arrowhead).

of patients. Other etiologies include trauma, structural anomalies, multisystem disease, drugs and toxins, viral infections, hereditary and metabolic disorders.[8] Pseudocysts are the most common complication of acute pancreatitis and usually occur after 4 weeks of developing acute pancreatitis. Most of the fluid occurs adjacent to the pancreas resolve, although some may be walled off by fibrous tissue and develop pseudocyst. Sonography and CT usually reveal single and unilocular cyst with a thick wall. Calcifications are rare. The fluid is hypoechoic on sonography and of water attenuation on CT. Fluid-debris level and internal echoes may be seen if there is infection or hemorrhage. Sign of pancreatitis is a useful clue of diagnosis. Drainage is required in case of large size and superimposed infection and surgery is the treatment of choice in case of bleeding or rupture.

Ovarian cysts

Frequently, ovarian cysts in the newborn and young child extend into the abdomen and present as an abdominal mass.[9] They can be detected prenatally by ultrasound or MRI. The cyst is stimulated by fetal, placental or maternal hormones. The management for the simple cysts that are smaller than 5 cm is observation in asymptomatic patients, because they will resolve spontaneously after hormone level decreases. However, the management for the cysts which are larger than 5 cm is quite controversial. If the patient has symptom or the cyst persists, surgery is recommended.[10] Complications include rupture, torsion and bleeding. When the fluid debris level is detected, preceeding ovarian torsion should be considered.[11]

Mature cystic teratoma (dermoid cyst)

Although most common site of teratoma is the sacrococygeal region and ovary, abdominal or retroperitoneal teratoma is not infrequent. Cystic teratomas are the common variety and usually benign. Sonographically, they manifest as the cystic mass with echogenic sebaceous material and calcifications. At CT and MRI, fat density and calcifications within the cyst are diagnostic. (Fig 3)

Duplication cyst (enteric cyst)

A gastrointestinal (GI) duplication cyst is a congenital anomaly which may occur anywhere along the GI tract and is attached to the GI tract. The distal ileum is the most common location. These cysts are lined with epithelium and have smooth muscle in its wall. They are usually close to the gastrointestinal tract, but do not communicate. Presenting symptoms depend on the location and size. A characteristic sonographic finding is presence of gut signature or double wall sign, inner echoic layer of mucosa and outer hypoechoic layer of muscle. (Fig 4)



Fig 3. Retroperitoneal mature cystic teratoma in a 1-year-old girl with abdominal distension. (A) Coronal T2-weighted MRI reveals a large cystic mass located in left sided retroperitoneum (arrow) displaced left kidney (K) superiorly. (B) Axial T1-weighted image shows hyperintensity of the fat (*) at superior aspect of the mass and (C) axial fat suppression image shows its hypointensity (*).

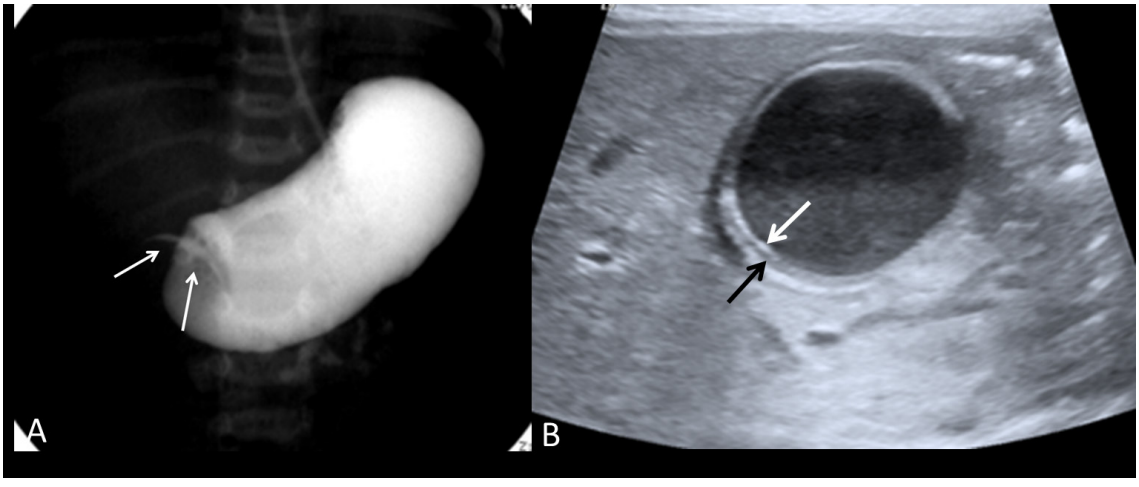


Fig 4. Duplication cyst of the stomach in a 7-day-old neonate with feeding intolerance and vomiting. (A) Upper gastrointestinal series demonstrate difficulty emptying of contrast and extrinsic compression at gastric outlet (arrow). (B) Ultrasonography demonstrates a cystic mass with gut signature: inner echoic layer of mucosa (white arrow) and outer hypoechoic layer of muscle (black arrow), located anteriorly to the pancreas.

Choledochal cyst

Choledochal cyst is one of the most common congenital hepatobiliary abnormalities and characterized by biliary tree dilatation. There are five types from Todani classification with different pathogenesis and appearance and type I is the most common. A minority of patients present with the triad of palpable abdominal mass, jaundice and fever. Sonography is usually the first imaging modality and demonstrates cystic mass at porta hepatis communicated with bile duct, which is the helpful clue. (Fig 5) It may contain stones or sludge. Surgical excision is the definitive treatment.

Adrenal hemorrhage

Adrenal hemorrhage usually occurs in newborn with birth trauma, hypoxia and sepsis. It is sometimes asymptomatic, but may present as palpable mass, anemia, or hypovolemic shock. Neonatal adrenal hemorrhage may be detected as cystic suprarenal mass due to liquefaction which overlaps with congenital cystic neuroblastoma. Sequential ultrasonography, CT or MR is helpful. The hemorrhage gradually decreases in size, changes echogenicity and eventually disappears or may calcify.[12,13] (Fig 6)



Fig 5. Choledochal cyst in a 27-day-old neonate with jaundice. Ultrasonography demonstrates dilated bile duct (arrow) emptying directly into the cystic mass (*) at porta hepatis containing echogenic debris.

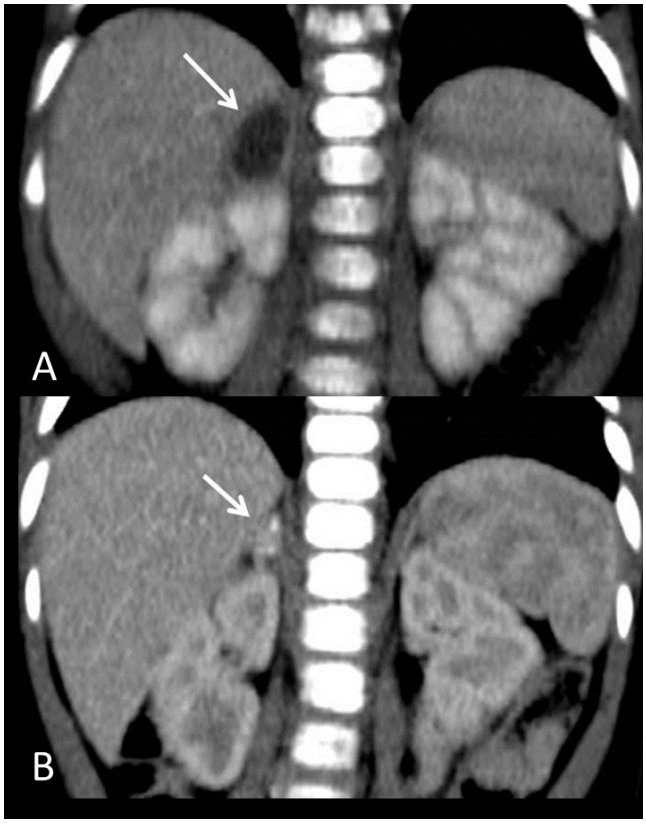


Fig 6. Adrenal hemorrhage in a 1-month-old neonate with jaundice. (A) Initial coronal CT shows a cystic adrenal mass (arrow). (B) Follow up CT demonstrates the mass resolved and was calcified (arrow).

CONCLUSION

There is a wide variety of cystic abdominal masses in infant and children. The most helpful factor in diagnosis is their location and imaging appearance. Fortunately, the majority in the pediatric age group are benign. Adrenal hemorrhage gradually decreases in size and disappears. Management of asymptomatic small ovarian cyst is observation. Most pancreatic pseudocysts resolve, but drainage and sometimes surgery are required when there are complications such as rupture, bleeding and infection. Surgery is the treatment of choice for omental and mesenteric cysts, dermoid cyst, choledochal cyst, duplication cyst and cystic teratoma and imaging can provide clues in diagnosis, determining the extension and guide surgical management.

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