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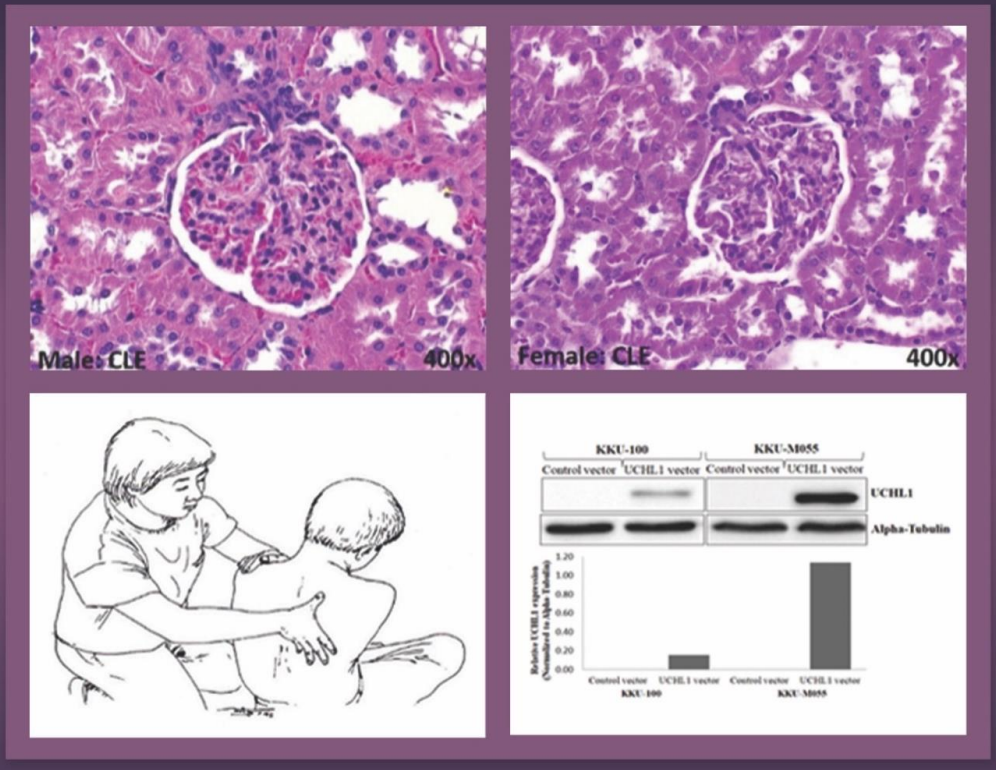

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ORIGINAL ARTICLE

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Promoter hypermethylation regulates UCHL1 expression in cholangiocarcinoma cell lines

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KEYWORDS
Hypermethylation; Silencing; *UCHL1*; 5-azacytidine.

ABSTRACT

Cholangiocarcinoma (CCA) is a significant health burden in Northeastern Thailand. Aberrant DNA methylation is a characteristic feature of most human cancers. Although overexpression of UCHL1 was observed in primary hilar CCA, the regulation of *UCHL1* expression by DNA methylation in CCA has not yet been confirmed. We herein detected DNA methylation and mRNA expression levels in CCA and immortalized cholangiocyte cell lines using MS-HRM and RT-PCR, respectively. The results showed inverse correlation between DNA methylation levels and mRNA expression levels in CCA cell lines. To confirm whether DNA methylation regulated *UCHL1* expression in CCA, 5-azacytidine was applied to inhibit DNA methylation process. We found that UCHL1 protein was up-regulated while DNA methylation was reduced after treatment suggesting the regulation of *UCHL1* expression by DNA methylation. To study the role of *UCHL1*, CCA cell lines were transfected with vector harboring *UCHL1* gene and control vector. Cell proliferation and chemotherapeutic drug sensitivity were investigated. The results showed no difference between these two groups. Our study reveals that overexpression of *UCHL1* alone is not sufficient to neither inhibit tumor cell growth nor enhance chemoresponsiveness. Nevertheless, *UCHL1* expression is epigenetically control indicating its function as a tumor suppressor by which the key features of *UCHL1* and its partners in CCA are worth investigating.

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Introduction

Cholangiocarcinoma (CCA) is a malignant tumor originating from the epithelial cells of bile ducts. The etiology of CCA is usually related to chronic inflammation and biliary duct cell injury induced by the obstruction of bile flow. Previous studies demonstrated that inflammation causes accumulation of aberrant epigenetic alterations[1-3].

DNA methylation is an epigenetic process which influences gene expression without altering the DNA sequence. This process arises from the transfer of methyl groups (CH_3) to the 5' position of cytosine nucleotides of CpG islands by DNA methyltransferases (DNMT). Normal DNA methylation levels are important for the development and function of healthy cells while abnormal DNA methylation levels may contribute to the initiation and progression of most cancers such as retinoblastoma[4], lung cancer[5], breast cancer[6], and CCA[7]. Global DNA hypomethylation may cause genome instability and DNA hypermethylation can lead to silencing of tumor suppressor genes[8,9].

UCHL1 (ubiquitin carboxyterminal hydrolase 1) is a deubiquitinating enzyme that plays role in protein stabilization[10]. The function of *UCHL1* is cleavage of conjugated ubiquitin (Ub) from targeted proteins. The role of *UCHL1* is controversial as there is conflicting evidence regarding its dual oncogenic or tumor suppressor properties depending on cancer type. For oncogenic function, UCHL1 has been shown to be overexpressed in breast cancer cells and promotes invasion[11]. UCHL1 stabilizes cyclin B1 through deubiquitination pathway and promotes cell cycle progression in uterine serous carcinoma[12]. In contrast, *UCHL1* is described as a tumor suppressor in pancreatic neuroendocrine tumors since loss of UCHL1 expression is associated with tumor metastasis. Aberrant DNA methylation of *UCHL1* is associated with prognosis of CCA patients. Nanok et al. (2018) demonstrated that the DNA methylation levels of *UCHL1* were associated with patients' overall survival[13]. CCA patients who had high methylation level of *UCHL1* exhibited significantly shorter survival than those who had low methylation. Similar result was also obtained in 5-fluorouracil-treated CCA patients. They speculated that loss of *UCHL1* expression was due to promoter hypermethylation resulting in the deregulation of *p53* function in apoptosis induction. Nevertheless, the effect of DNA methylation on *UCHL1* expression in CCA has not yet been confirmed. Hence, we aimed to address whether DNA methylation regulated *UCHL1* expression in CCA cell lines. Moreover, the role of UCHL1 in cancer is still unclear, for example, ectopic expression of *UCHL1* promotes proliferation in colorectal cancer cells and tumor volume in xenograft mice[14]. On the other hand, ectopic expression of *UCHL1* significantly reduces the colony formation and proliferation of breast cancer cells via G0/G1 cell cycle arrest and apoptosis induction[15]. Accordingly, in this study, we aimed to elucidate the role of *UCHL1* in CCA cell lines.

Materials and methods

Cell lines and cell culture

Intrahepatic CCA cell lines (KKU-100 and KKU-M055) were established in the Cholangiocarcinoma Research Institute, Khon Kaen, Thailand[16]. Immortalized cholangiocyte cell line (MMNK-1) was established and characterized at Okayama University[17]. Cells were cultured in Dulbecco's Modified Essential Medium (Gibco-BRL, Ontario, Canada) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco-BRL) at 37 °C in a 5% CO_2 atmosphere.

DNA extraction and Bisulfite modification

DNA from cell lines was extracted using Qiagen® Blood & Tissue kit (Qiagen, Hilden, Germany) and quantitated by spectrophotometry at 260 nm. One microgram of DNA was treated with bisulfite using EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. The modified DNA should be used immediately or stored at -20 °C until it was used as a template for methylation sensitive-high resolution melting (MS-HRM) analysis.

Methylation-sensitive high resolution melting (MS-HRM)

The specific primers of CpG islands related to promoters of *UCHL1* gene were performed as described by Nanok et al. (2018)[13]. PCR amplification and HRM were performed on a Rotor-Gene Q (Qiagen, South San Francisco, CA, USA). The reaction mixture was performed in a final volume of 50 µL containing 50 ng of bisulfite-modified DNA, 1X PCR buffer (67 mM Tris, pH 8.4, 16.6 mM ammonium sulfate and 0.1% Tween 20), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 300 nM of each primer, 1.5 µM SYTO®9 (Invitrogen, Carlsbad, CA, USA), 0.5 unit of Platinum Taq DNA polymerase (Invitrogen). The amplification steps were composed of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. HRM step consisted of reannealing at 60 °C for 90 sec and slowly warmed by continuous acquisition to 99 °C with rising by 0.5 degree each step. The standard of DNA methylation including 100, 75, 50, 25, 10, and 0% were obtained by mixing bisulfite modified fully methylated (100%) and unmethylated DNA sequences. The HRM data were analyzed using Rotor-Gene Q Series Software 2.1.0 (Qiagen). The value of differential fluorescence of each methylation control against 0% methylation was used to generate a standard curve. The linear equation of each MS-HRM was performed in Microsoft Excel 2010 and used for quantification of methylation levels.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell lines using SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). The cDNA was synthesized by reverse transcription of RNA template using ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's protocols. The specific primer sequences were as follows; *UCHL1*: forward 5'- CTGGGATTTGAGGAT-GGATC -3'; reverse 5'- GTCATCTACCCGACATTGGC -3'. The 50 µL of PCR reaction consisted of 1X PCR buffer (67 mM Tris, pH 8.4, 16.6 mM ammonium sulfate and 0.1% Tween-20), 0.2 µM of each primer, 200 µM of each dNTP, 50 ng of cDNA, 1.5 µM SYTO®9 (Invitrogen), 2 mM $MgCl_2$ and 0.5 unit of Platinum Taq DNA polymerase (Invitrogen). The cycling stage was performed as the following steps: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 1 min. The PCR reaction was carried out using a Rotor-Gene Q (Qiagen). Relative gene expression was analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$)[18].

5-azacytidine treatment

The 5-azacytidine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO and prepared as 10 mM stock solution. CCA cell lines with density of $2x10^5$ were seeded in 6-well plates with cultured medium 2 mL/well. Cells were incubated at 37 °C with 5% CO_2 condition for 24 h, after which 2 mL of fresh cultured medium containing 1 µM of 5-azacytidine or 0.01% DMSO control were replaced for every 24 h until 5 days.

Transfection

CCA cell lines with density of $5x10^5$ cells were seeded in 6-well plates (80% confluence). Then, cells were transfected with 2.5 µg of plasmid harboring *UCHL1* gene, pcDNA3.1+/C-(K)DYK vector (GenScript, Piscataway, NJ, USA) and 2.5 µg of control vector using 3.5 µL of lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. After incubation for 24 h, transfected cells were trypsinized and used for further analysis.

Western blot analysis

CCA cell lines were lysed in 1X RIPA Lysis buffer (Millipore, Darmstadt, Germany) containing protease and phosphatase inhibitor. Total protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). Thirty micrograms of protein samples were separated on a 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. After non-specific block with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), membranes were incubated overnight at 4°C with primary antibodies against UCHL1 dilution 1: 1,000 (Cell signaling #3524, Beverly, MA, USA) or alpha-tubulin dilution 1: 10,000 (Cell signaling

#2144S). Membranes were washed with TBS-T, incubated at room temperature with goat anti-rabbit IgG dilution 1: 5,000 (Cell signaling #7074) for 1 h, and signal was detected using ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences, Chicago, IL, USA).

Cell proliferation assay

Cell proliferation was measured by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). Briefly, 5,000 cells were seeded to each well of a 96-well plate. After incubation for 24, 48, and 72 h, MTS solution (Promega) was added to each well and further incubated at 37 °C with 5% CO_2 for 2 h before absorbance is being measured at 490 nm using a Synergy HTX (BioTek, Winooski, VT, USA).

Cytotoxicity assay

Cells with density of 3,000 were seeded in triplicate in a flat-bottom 96-well plate and allowed to grow for 24 h. Then, 100 µL of medium containing different concentrations of gemcitabine were added to each well at the concentration of 0, 10, 20, 40, 80, and 160 nM, respectively. After 72 h, cell viability was performed using MTS assay. Moreover, 100 µL of medium containing different concentrations of cisplatin were added to each well at the concentration of 0, 0.25, 0.5, 1, 2, and 4 µg/mL, respectively. After 48 h, cell viability was performed using MTS assay.

Statistical analysis

The statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The comparison of methylation levels, gene expression, cell proliferation, and cytotoxicity assay were analyzed using Student's t-test. *p*-value < 0.05 was considered statistically significant.

Results

UCHL1 is highly methylated and lowly expressed in CCA cell lines

We used MS-HRM for analyzing the DNA methylation levels of the *UCHL1* promoter in CCA and immortalized cholangiocyte cell lines (MMNK-1). The results showed that DNA methylation levels were significantly high in KKU-100 and KKU-M055 when compared to MMNK-1 (Figure 1A). This is consistent with the relative mRNA expression of *UCHL1* that was abundantly expressed in MMNK-1 cell line, but silenced in CCA cell lines (Figure 1B).

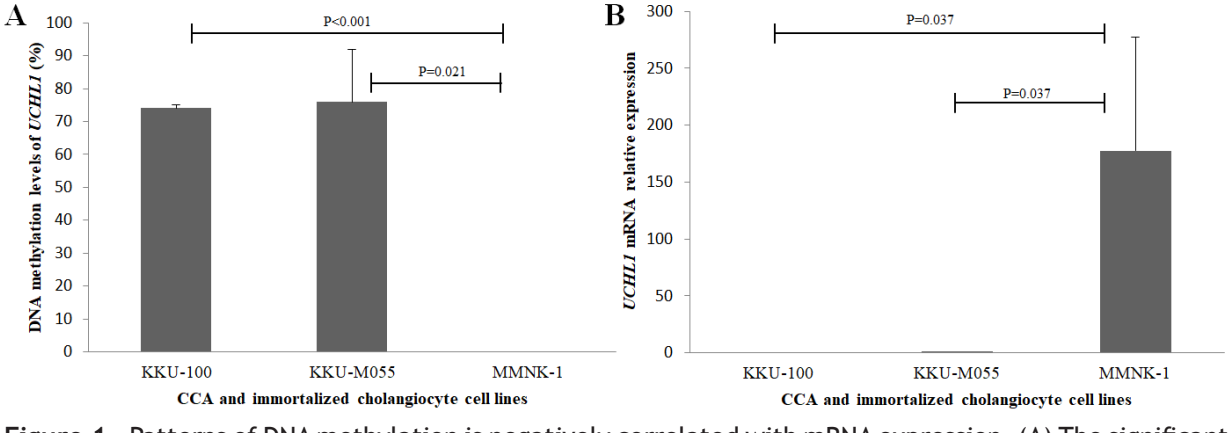

Figure 1 Patterns of DNA methylation is negatively correlated with mRNA expression. (A) The significant differences in DNA methylation between CCA cell lines and MMNK-1. (B) The significant differences in mRNA expression between CCA cell lines and MMNK-1.

Upregulation of UCHL1 in CCA cells following demethylation

To address whether DNA methylation was the cause of *UCHL1* silencing, KKU-100 and KKU-M055 CCA cell lines were treated with the demethylating agent, 5-Azacytidine. As a result of treatment, CCA cell lines showed the significant reduction of DNA methylation levels (Figure 2A) and restoration of UCHL1 protein in KKU-M055 (Figure 2B). These results suggested that DNA hypermethylation in the promoter region of *UCHL1* is responsible for its transcriptional silencing.

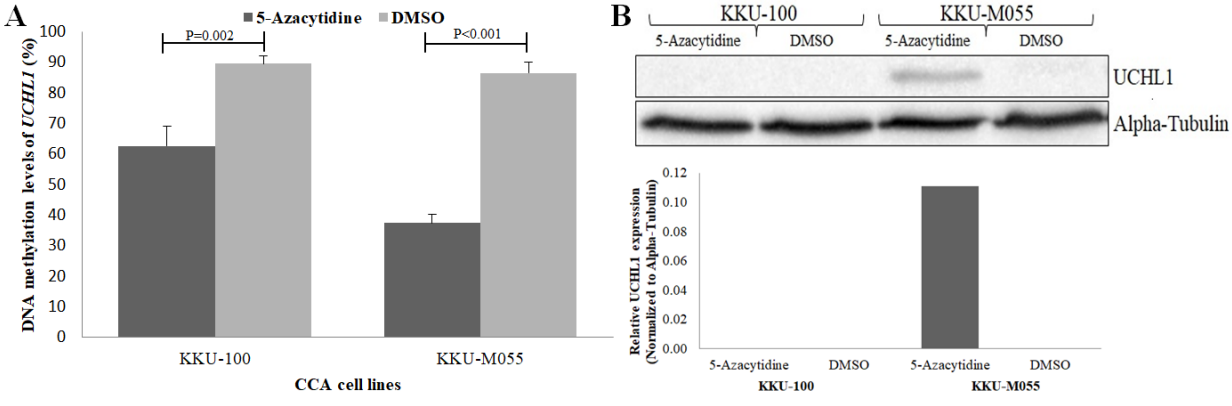

Figure 2 Effects of 5-azacytidine on DNA methylation and protein expression. (A) DNA methylation levels of *UCHL1* in KKU-100 and KKU-M055 were significantly reduced and (B) UCHL1 protein was re-expressed in KKU-M055 after 5-azacytidine treatment.

Re-expression of UCHL1 protein in CCA cell lines and the effect of UCHL1 overexpression on cell proliferation and chemotherapeutic drug sensitivity

Due to the low expression of *UCHL1* in CCA cell lines, we transfected UCHL1 vector into CCA cell lines to restore UCHL1 protein and study the effect of *UCHL1* on cancer phenotype while pcDNA3.1+/C-(K)DYK vector was used as a control. As shown in figure 3A, the transfection was successful as UCHL1 protein was expressed in CCA cell lines. Since the level of UCHL1 protein expression in KKU-100 was low; therefore, we used only KKU-M055 for further studies.

To address whether *UCHL1* played a role in anti-proliferation in CCA, MTS was performed in KKU-M055 transfected cells. The result showed that the overexpression of UCHL1 could not suppress the proliferation of KKU-M055 (Figure 3B). To address whether *UCHL1* played a role in chemotherapeutic drug sensitivity, KKU-M055 transfected cell was treated with various concentrations of gemcitabine for 72 h and cisplatin for 48 h, then MTS assay was performed. The result showed that *UCHL1* had neither effect on gemcitabine (Figure 3C) nor cisplatin (Figure 3D) sensitivity.

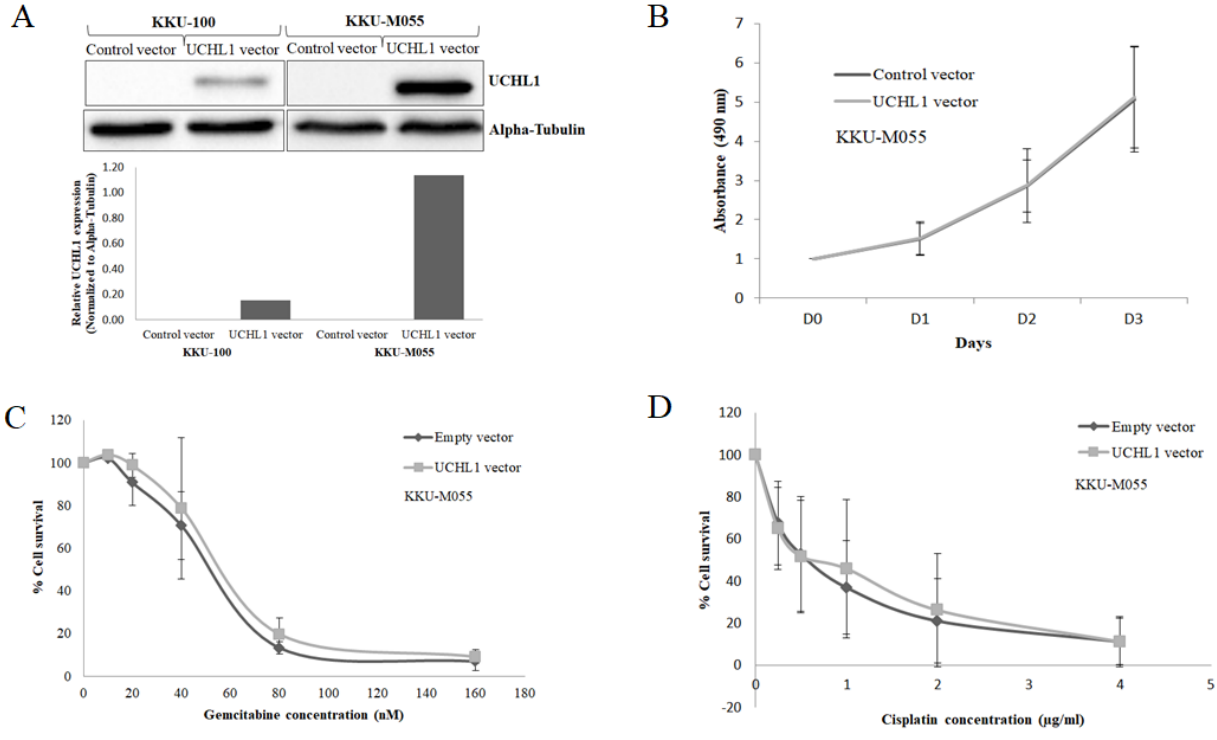

Figure 3 Effect of *UCHL1* overexpression on cell proliferation and chemotherapeutic drug sensitivity. (A) Transfection of the UCHL1 vector into CCA cell lines. The effect of UCHL1 protein overexpression on (B) Cell proliferation, (C) Gemcitabine sensitivity, and (D) Cisplatin sensitivity.

Discussion

Aberrant DNA methylation in cancer has been observed in specific genes. One of them is *UCHL1* which has been reported to be silenced by promoter hypermethylation in many types of cancer such as breast cancer[15], prostate cancer[19], renal cell carcinoma[20], colorectal cancer[21], ovarian cancer[22] and nasopharyngeal carcinoma[23]. These results were consistent with our studies which high methylation of *UCHL1* and low expression of this gene were found in CCA cell lines, indicating that DNA methylation is the major cause for *UCHL1* silencing in CCA cell lines.

Moreover, our study revealed that *UCHL1* was re-expressed by epigenetic drug which was consistent with the study of Xiang et al. (2012)[15]. Although DNA methylation level was significantly decreased in KKU-100 after 5-azacytidine treatment, UCHL1 protein could not be detected. This finding suggested that 5-azacytidine could demethylate *UCHL1* in CCA cell lines but KKU-100 may require either higher dose or longer time treatment than KKU-M055 to restore UCHL1 protein because its doubling time was much longer than that of KKU-M055 (72 h *vs* 25 h)[16,24]. An increase of methylation level observed in KKU-100 and KKU-M055 after treatment with DMSO (Figure 1A VS Figure 2A) was evident by the study of Yokochi and Robertson (2004)[25]. They showed that DMSO stimulates catalytic activity of *de novo* DNA methyltransferase 3a (DNMT3a) *in vitro*.

Functionally, overexpression of *UCHL1* has been found to suppress cell proliferation in breast cancer cells[15] while knockdown of *UCHL1* has been found to reduce the reversal effect of verapamil on chemoresistance to Adriamycin (Doxorubicin trade name) in hepatocellular carcinoma cells[26] and the increase of cisplatin resistance in ovarian cancer cells[22]. Moreover, previous study has shown the association between

UCHL1 protein and TP53 by which UCHL1 protein stabilizes TP53 from proteasome degradation. The study of Li et al. (2010) demonstrated that UCHL1 protein could activate the TP53 signaling pathway by deubiquitinating TP53 and $p14^{ARF}$ protein. The stable $p14^{ARF}$ protein has been the cause of MDM2 inhibition and TP53 accumulation[23]. In our study, no significant alteration of cell proliferation property and chemotherapeutic drug sensitivity were found in *UCHL1* overexpressing CCA cell line. The IARC TP53 Database showed that most of CCA cell lines harbor *p53* mutation. Hence, if the impact of *UCHL1* on cell proliferation and chemosensitivity was influenced by *p53* signaling, it was reasonable for our result that overexpression of UCHL1 in CCA cell line was unable to affect a change in cancer phenotype. Furthermore, some tumor suppressor genes which were stabilized by UCHL1 protein activity may also have promoter hypermethylation resulting in gene silencing. Chinnasri et al. (2008) showed that DNA methylation is the main mechanism of $p14^{ARF}$ inactivation in CCA leading to the loss of $p14^{ARF}$ protein expression[27]. If TP53 was indirectly controlled by UCHL1 via $p14^{ARF}$/*MDM2*/*p53* pathway, it would lead to the results with unchanged cancer phenotype after overexpression of *UCHL1*. Therefore, combined overexpression of *UCHL1* and its targeted genes may lead to significant change in cancer phenotype. To clarify this point, combined overexpression of *UCHL1* and its target, $p14^{ARF}$, in CCA cell lines expressing wild type *p53* will lead to TP53 accumulation contributing to apoptotic induction when treated with chemotherapeutic drug.

Conclusion

We have shown that the expression of *UCHL1* in CCA is regulated by DNA methylation and UCHL1 only by itself is robustly unable to play a crucial role in CCA; it must work in concert with other active molecules to be actionable.

Take home messages

DNA methylation plays a key role in UCHL1 expression.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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ORIGINAL ARTICLE

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The commercial value and viability of a non-invasive knee cartilage injury detection device

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KEYWORDS
Knee osteoarthritis; Early detection; Economic valuation; Medical device; Medical technology.

ABSTRACT
Knee osteoarthritis (OA) is becoming more common, and is affecting more young population than before. Symptoms including pain and stiffness become present only when the injury is irreparable, and the most effective treatment is surgery. The ability to detect the injury early can prevent the disease progression to that stage with appropriate interventions, saving patients from physical suffering as well as financial cost. This paper assesses the commercial value of such a device that can detect early stages of knee OA injury. The information is useful for private investors as well as policy makers in setting funding priorities. We used questionnaires to collect willingness-to-pay data from 400 respondents in Khon Kaen municipality, and used this data to make revenue projections for a provider of knee injury reading service. We found that the revenue is substantial even for smaller operations like clinics. The willingness-to-pay for a device is about 190,000 THB per year for a small clinic requiring 60% operating profit margin, and goes up to 7.89 million THB per year for a large hospital requiring 30% profit margin. We conclude that if device developers can keep their costs below this willingness-to-pay, an early detection device for knee injury is commercially viable and can reach a wide population.

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Introduction

The ability to walk is arguably the most symbolic aspect of a person's independence, and it significantly lightens the burden on caretakers, e.g. for the elderly, for those living dependently. Knee osteoarthritis causes pain, aching, and stiffness, all of which affect their ability to walk[1]. Those with knee OA are limited in their daily functions[2], becoming heavier burdens on care-takers if dependent. The most effective treatment for knee OA is surgery, which is expensive and out of reach for lower income patients. The prevention of disease progression to surgery stage would improve the well-being and reduce the financial burden of a large part of the population.

Symptomatic knee OA is characterized by progressive articular cartilage deterioration and resulting in pain, aching or stiffness in the knee[1] affecting quality of life and psychological well-being[3]. Risk factors of OA include old age, female gender, obesity, previous knee injury, repetitive use of the knee joint, high bone density, muscle weakness and joint laxity[8]. Knee cartilage injury can also be found in asymptomatic young individuals. A recent study has reviewed the knee MRI of 230 asymptomatic individuals and found 62% with knee cartilage injury, where approximately 20% were 40 years old or younger[9]. In another study done in a larger population pool, 24% of asymptomatic individuals developed knee cartilage injury[10]. This asymptomatic group is likely to later develop symptomatic knee OA.

When diagnosed with knee OA, physicians typically prescribe conservative therapy, which includes both pharmacological and non-pharmacological methods. Non-pharmacological therapy includes, but is not limited to, weight loss for the overweight or obese, exercise, physical therapy, cane use, and knee bracing[2,11]. Pharmacological approach includes the prescription of oral or topical nonsteroidal anti-inflammatory drugs (NSAIDs) for pain relief, and intra-articular glucocorticoid injection[12]. However, NSAIDs are associated with upper gastrointestinal irritation for non-selective NSAIDs and risk of cardiovascular disease for selective NSAIDs[13].

Osteoarthritis (OA) was estimated to affect approximately 14 million people in the United States, where the disease affects not only the elderly but also younger and middle-aged adults[1]. Knee OA is becoming more common among the younger population[3]. In Thailand, according to data provided by the Thai National Health Statistics Office, the number of knee replacement surgeries performed in 2016 (B.E.2559) among those under the Universal Coverage (UC) health insurance scheme amounts to more than 11,000[7]. The total number of surgeries is likely substantially higher as these count only those who cannot afford the surgery on their own. The ability to detect the injury early can help patients avoid surgeries and allow physicians to develop treatments for various stages of the disorder.

OA might be preventable as there are several risk factors other than old age[14]. Weaker quadriceps in radiographic OA[15] suggests muscle strength training could further protect the knee beyond weight loss. Knee joint screening is currently not on the list for annual physical checkup. At present, MRI is arguably the most powerful tool in detecting many pathologies including knee cartilage injuries[4]. However, the machine is expensive and out of reach for most health care service providers[5]. The current rate for an MRI of the knee is about 7,200 THB, which is more than half the current average salary of Thai wage workers of about 14,000 THB in 2016[6]. An effective and less costly screening device to assist in knee OA prevention seems likely generate health benefits reaching a broader population.

The case of the MRI illustrates that the ability of any health technology to improve lives depends on the ability of the private sector to build a business around it, offering a price that is within reach. That ability depends, first and foremost, on the revenue potential of the technology. Hence, this paper provides estimates of revenue potential of a knee cartilage injury detection device (KIDD) in Thailand, as a less expensive and more accessible tool for early detection of knee injuries. We use the contingent valuation method (CVM) to elicit willingness-to-pay for knee injury reading service, and assess the

commercial viability of offering the service and of making such device available. As of the writing of the paper, there is no standard practice to treat early stages of knee cartilage injury, as most are undetected.

Materials and methods

The assessment of demand for, and commercial viability of, the knee injury reading service depends on estimating the value of a non-traded item, since it is not available in the market. This study applied the contingent valuation method (CVM) to assess the value of the device to potential customers. The method involved asking the target group the question "how much would you pay for this service?" or "will you accept the service at this price?" and then offer a list of prices[16]. We used results from CVM to project the revenue for providers of knee injury reading service, and deduce the commercial viability based on these projections.

Demand assessment using Contingent Valuation Method (CVM)

We used the Contingent Valuation Method (CVM) to estimate the willingness-to-pay for a knee injury reading service. This method is regularly applied in the medical context to find the value of a service not traded in the market. See van den Berg, Brouwer, & Koopmanschap (2004) for an overview of this and other methods[16].

After identifying the target market, we asked people whether they were willing to accept the service at various prices. This was to replicate purchase decisions in real life where consumers look at the posted prices to determine whether to buy the product. The data gathered from the surveys were then aggregated to estimate the demand curve relating price to quantity purchased.

Since survey participants were not familiar with knee injury and early detection, data collectors provided the following information before conducting the survey.

1) The importance of healthy knees in living a full life

2) Current knee injury detection practice

3) Knee injury detection using non-invasive device

The above information was also available on the questionnaire for respondents to read and reconsider throughout the interview process. Respondents could also have the interviewers read the information to them. In this study, none of the participants needed assistance.

The CVM questionnaire was the main tool of analysis, and biases could occur if it is not carefully designed. We considered and addressed 3 main sources of error where our instruments might be biased, following the literature[17]. These were 1) understanding the knee reading service and its importance, 2) correctly stating their willingness to pay, and 3) the representativeness of the data. We consulted with a fellow researcher with experience in CVM to help with the information presentation, and pre-tested the material by having colleagues and research assistants read the information and provide feedback. In regards to the second source of bias, we designed the question as yes/no (binary) at given prices, which should mimic real world purchase situations better than asking them to state a price. For representativeness of the data, we randomly selected participants who fell within the target age range. This latter point was discussed in more detail below.

Survey sample

The sample consisted of people aged 18 - 40 from Khon Kaen Municipality (Amphoe Mueang) in Thailand's Northeast region. The selected age group was in accordance with knee treatment experts who indicated that they would likely target the population between the ages of 18 - 30 for early knee injury detection and prevention. The research team would visually guess the age of each passerby, and verbally recruited one out of about 2-3 of those who seemed to meet the age criterion. Recruitment of respondents took place at crowded locations where the target group were most likely to be found. These included locations at a university campus and a public park near the city center. Since the average income in the Northeast is lower than that in Bangkok or the

central region, the estimated willingness to pay can be lower. We were able to collect data from 400 respondents. Females made up about 53% of the sample. Summary statistics of the respondents are given in the table below.

Table 1 Summary statistics of survey respondents

Variable	Observations	Values (SD)
Age (years)	400	24.01 (6.01)
Monthly income (Thai Baht)	282	9,598.40 (7978.40)
Female (%)	400	52.5%
College Graduate (%)	400	38.5%
Occupation		
Student (%)	400	65.5%
Government worker (%)	400	18.5%
Private sector worker (%)	400	5.3%
Agriculture worker (%)	400	3.5%
Self-employed (%)	400	2.3%

Note: Source from authors' calculations

The respondents did not show obvious physical disabilities. The income level of the sample was about 4 times the regional poverty line of 2,417 THB in 2018[18]. The sample was not screened for any medical condition, nor were they asked about existing health issues. The majority of respondents were college students, since the city was home to a major regional university and a few other technical colleges. A different sample consisting of more working adults than students would likely give different results, even if they came from the same age group.

The survey asked respondents to answer either "yes" or "no" to accepting knee injury reading once during the coming year at various prices. The prices ranged from 50 to 2,000 baht for checking both knees. We solicited willingness-to-pay information for two scenarios. In scenario 1, the service requires required a one-week advanced appointment. Scenario 2 was the same except there was no advanced appointment necessary. With binary responses, the sample size was calculated as[19].

$$n \geq \frac{t^2 pq}{d^2}$$

The sample size, n, was determined by the acceptable t-statistics, t, based on the acceptable risk of a type-I error (or statistical significance), α, the proportion choosing each of the binary options, p and q, and an acceptable margin of error for the proportion, d. In this study we chose an acceptable margin of error of 5%, an α level of 0.05, and assumed $p = q = 0.5$. This resulted in a sample size of $n \geq \frac{1.96^2(0.5)(0.5)}{0.05^2} = 384$ respondents.

Results

From the questionnaire responses, we have data on people's decision to accept or reject a knee injury reading at various prices. This information is used to calculate the average willingness-to-pay for a knee injury reading, the optimal service price, revenue projections, and commercial viability. It is important to note that the sample used in this study is not representative of the market for any particular potential service provider such as a clinic or hospital. The selected

age range is likely younger than patients that most clinics or hospitals receive, and their willingness to spend on health services is likely lower. Their student status likely makes their willingness-to-pay even lower than in the working population. Thus, we view these results as conservative (lower bound) estimates of willingness-to-pay, optimal price, and revenue projections.

Willingness-to-pay

The overall average willingness-to-pay for a knee injury reading service with 1-week advance appointment is 761.31 THB (SD = 561.85, n = 389), and is 1,019.52 THB (SD = 625.80, n = 392) without an advance appointment. These are computed as the average of the maximum price where respondents still accept the service. We lose about 10 observations for these calculations, because some respondents report not willing to get a reading even if it is free, implying that they are not in the relevant market and are thus not considered in the willingness-to-pay estimation. We can see that the appointment period has some effect in reducing people's willingness to pay for the service. This illustrates that people consider not only financial but convenience and time costs in obtaining the service.

Service price and revenue

It is instructive to show the additional revenue per patient per year from offering knee injury reading service. This is computed as

revenue per patient = % taker × price

The first term, % taker, is the percentage of all patients who accept the knee injury detection service at the offered price. The additional revenue is on a patient-year basis. They will apply only ONCE to patients that visit the service provider, regardless of how many times they visit after that. Thus, these are revenues per first-visit patient at the service provider, referred to as "new patients" for conciseness. The total revenue generated is this number multiplied by the total number of new patients. The table below summarizes the additional revenue per patient generated from offering the service at each price.

Table 2 Additional revenue per patient for knee injury reading provider

Price offered	1-week appointment		No appointment	
	% taker	Revenue per new patient (baht)	% taker	Revenue per new patient (baht)
0	97.0%	0.00	97.3%	0.00
50	96.0%	48.00	97.3%	48.63
100	90.8%	90.75	96.3%	96.25
200	79.5%	159.00	89.3%	178.50
400	61.3%	245.00	75.5%	302.00
600	40.5%	243.00	60.3%	361.50
800	30.0%	240.00	47.0%	376.00
1000	22.0%	220.00	35.0%	350.00
1500	7.3%	108.75	20.3%	303.75
2000	4.5%	90.00	14.3%	285.00

Note: Source from author's calculations

The maximum additional revenue per new patient under each scenario is highlighted, as well as the corresponding prices. In the case of a required 1-week appointment, the price that maximizes additional revenue per first-visit patient is 400 baht per reading, taken up by 61.3% of patients, and the provider generates 245 baht per patient in additional revenue. Under no-appointment, the price is 800 baht, taken by 47% of patients, generating 376 THB additional revenue per patient visiting the provider for the first time in the course of a year.

The actual revenue a service provider will generate depends on the age and income characteristics of their typical mix of patients, since age is found to have a negative effect while income has a positive effect. Multiple Regression estimates (not reported here) show that an additional year in age is associated with about 20-30 THB lower willingness-to-pay, while an additional 1,000 baht in monthly income is associated with about 26-27 THB higher willingness to pay.

Revenue projections

Total revenue will depend on the total number of service recipients. As with most new technology, there will likely be an S-curve life cycle for the number of users[20]. This would then translate to a revenue trajectory that rises and then falls over time. In this work we assume a constant revenue stream that ends abruptly at the end of the cycle, which is assumed to be several years. We make projections on a yearly basis to avoid making careless assumptions on the length of this cycle. The constant-revenue assumption understates the importance of increased popularity towards the middle of the cycle, and overstates the use rate at the beginning and at the end. Without knowledge of their relative strength, we assume that these balance out and therefore use the constant revenue model and compute revenue streams per year.

According to a practicing physician we spoke to, about 20 - 30 new patients per day is plausible for clinics. This translates to about (20,30)/day x 365 days = 7,300 – 10,950 non-repeat patients per year. For hospitals, the estimated number of visit is 50 to 70 patients per day. The annual non-repeat visits for hospitals will thus be about 18,250 to 25,550 non-repeat patient visits per year.

The resulting projections of additional revenues generated are shown in table 4 for various new visit scenarios, using the no-appointment optimal price (800 baht) that generates 376 baht/new-visit in additional revenue.

Table 3 Annual revenue scenarios for various numbers of new visits per day

New visits per day	Total annual revenue (baht)
5	686,200
10	1,372,400
25	3,431,000
50	6,862,000
100	13,724,000

Note: Source from author's calculations from questionnaire responses

Commercial viability for a producer of knee injury detection device

The above results show that a clinic serving 25 non-repeat patients per day can expect to earn about 3.4 million baht in additional revenue per year by offering knee injury reading service. More traffic would generate more revenue. Private hospitals receiving about 50 new visits per day can generate 6.8 million THB per year. To determine the viability of a business that produces and sells and/or licenses the use of a knee injury detection device, it is important to know the potential

revenue they can generate. The willingness-to-pay of service providers for the device can give this information.

The revenue that service providers generate represents theoretical upper bounds of the willingness to pay per year for the device. The true willingness to pay will also account for cost and required profit margin. The cost of service will vary across establishments, and is likely lower per unit for larger operations due to economies of scale. A larger cost and required profit margin will reduce the willingness-to-pay for the device.

We assume a constant cost per reading of 100 baht, which includes overlays as well as other variable costs. Under the no-appointment scenario, where the optimal price to charge is 800 baht the willingness of clinics and hospitals to spend per knee injury reading is

$$800(1\text{-profit margin})-100$$

With these assumptions, we can then compute the hypothetical willingness to pay of clinics and hospitals for obtaining a knee injury detection device for 1 year, per non-repeat patient. Under the no-appointment scenario, 47% of all patients (0.47) accept the reading at the price of 800 baht. Thus the willingness of clinics and hospitals to pay, per non-repeat patient is

$$(800(1\text{-profit margin})-100)\times 0.47$$

The highest profit margin that still allows positive willingness to pay from providers is 87.5%. For illustration, the table below shows the willingness-to-pay per patient at profit margins of 30% and 60% of the price, under the no-appointment case (800 baht per reading, or 376 baht per non-repeat patient) for various targets of profit margin.

Table 4 Willingness of clinics and hospitals to pay for KIDD per new visit

Profit margin (% of revenue per new visit)	Profit per new visit (baht)	Willingness to pay per new visit (baht)
30	112.8	216.2
60	225.6	103.4
87.5	329	0

With the information from the table, we can compute total willingness to pay in hypothetical cases for service providers. For illustration, the table below provides results under 30% and 60% profit margins.

Table 5 Willingness to pay for knee injury detection device for one year (million baht)

Profit margin	New visits per day				
	5	10	25	50	100
30	0.39	0.79	1.97	3.95	7.89
60	0.19	0.38	0.94	1.89	3.77

The willingness to pay for the device ranges from 0.19 million baht per year in a clinic that expects 5 new visits per day and a 60% profit margin on additional revenue per patient, all the way to 7.9 million baht per year for a provider expecting 100 new visits per day and a 30% profit margin. There certainly seems to be room for buyers and sellers to meet to agree to a price that benefits both parties. Manufacturers and/or contractors can aim at keeping their costs of manufacturing and maintenance of the device within this range.

Discussion

The above results can inform business decisions as they provide estimates of potential revenues to be generated. Manufacturers and/or contractors can aim to produce the device or provide the service at the cost under the willingness-to-pay. They should also consider alternatives available to clinics and hospitals for knee injury testing, which will tend to lower the price consumers are willing to pay and thus the price they can charge and the ensuing revenue. A manufacturer of the device might also consider research institutions and sports teams as potential customers, in addition to clinics and hospitals as outlets for their product. These establishments will likely have their own willingness-to-pay based on different sets of factors, and more accurate estimates of potential revenue will require further investigation.

The estimates also show the significant value that people place on early detection of knee injury. Health policymakers might use this as indication of the social value that can be generated if a device or technology is available to serve this purpose, and set funding priorities accordingly. In addition, if such a cheap, non-invasive device becomes available, it might help some patients to avoid surgery as they consult with their physicians on how to approach the injury. At the same time, the measurement allows a collection of data on knee OA and its progression, across various segments of the population, which is essential for research to advance the science of treatment and prevention.

An important shortcoming in these estimates of the willingness to pay is in the use of questionnaires, where respondents may behave differently in real life. This is a major limitation of the so-called "stated-preference" method that tend to overstate willingness-to-pay compared to the "revealed-preference" method where actual behavior is observed[21,22]. Furthermore, respondents are framed to consider only the one decision at hand, which is whether to accept the service at particular prices. Different types of framing or presenting the service can affect real usage[23]. However, revealed-preference and alternative framing can be costly to carry out, and are appropriate when more funding is available and the stakes are higher[17].

Finally, the commercial viability assessed here assumes that the system can generate a business to capture the willingness-to-pay of both customers and service providers. This is a very strong assumption, as businesses, especially startups with their high-risk and high-reward nature, routinely fail[24]. Issues like promotions, customer relations, product design, production, supply chain, and even labor relations all contribute to a successful business. Without these in place, the values that customers are willing to pay cannot be translated to business income as businesses fail to capture them.

Conclusion

Early detection of knee cartilage injury is of medical value as it allows for early treatment of the condition as well as evidence-based study of its progression. If available, a wide of the device can improve the health outcomes of those at risk of knee OA, which is a growing population. The results in this study show substantial potential for commercial value as well, where the plausible range of service price seems sufficient to generate profit for potential manufacturers as well as service providers.

Take home messages

Offering knee cartilage injury reading can generate substantial revenue from the target population, both in small scale and in large scale. This translates to a sufficiently high willingness-to-pay of service providers for a knee injury reading device. Research and development of the device is likely to generate a satisfactory return. The service can reach a wider segment of the population than the current technology if priced appropriately, which will contribute to improvement in treatment practices and health outcomes of knee OA patients.

Conflicts of interest

The authors declare no conflict of interest.

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Cognitive function and arterial stiffness in overweight and obese diabetes compared with non-obese diabetes individuals

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KEYWORDS
Obesity;
Type 2 diabetes;
Arterial stiffness;
Cognitive function.

ABSTRACT

Arterial stiffness is commonly observed in type 2 diabetes (T2DM) patients and may explain the cognitive dysfunction. Obesity is associated with T2DM; however, the additive effects of overweight and obesity on cognitive function and arterial stiffness have not been investigated. The aim of this study was to determine whether the concomitance of obesity and T2DM cause a further impairment of arterial stiffness and cognitive function. Arterial stiffness and cognitive function were measured in sedentary healthy subjects, T2DM with normal body mass index and T2DM with obesity patients. Carotid- femoral pulse wave velocity (cfPWV) and brachial ankle PWV (baPWV), measures of central arterial stiffness, were higher (p-value < 0.05) in diabetes with obesity and diabetic patients than in sedentary controls. There was no difference in central and peripheral arterial stiffness between normal weight and obese T2DM groups. The Montreal Cognitive Assessment (MOCA) score was higher (p-value < 0.05) in diabetes with obesity than in diabetic patients and sedentary controls. In conclusion, diabetes with obesity showed the cognitive function higher than diabetic patient with normal weight. The level of arterial stiffness was not related to cognitive function in diabetic with obesity patients.

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Introduction

Type 2 diabetes mellitus (T2DM) is one of the cardiovascular disease risks. Because of the high prevalence, diabetes is certain to be the most challenging health problem worldwide in the 21st century. The strong evidence revealed the epidemiological association between T2DM and obesity. Obesity amplifies the risk of cardiovascular disease (CVD) in T2DM because of poorer glycemic control, blood pressure, and lipid profiles[1].

In light of high numbers of elderly population, the burden of cognitive dysfunction becomes increasingly for health care resources. Cardiovascular risk factors are important predictors of cognitive decline and dementia. Cognitive dysfunction is an important complication of T2DM. Compared to the general population, people with diabetes have a 1.5 fold greater risk of cognitive decline and a 1.6 fold greater risk of future dementia[2]. Obesity is also associated with a cognitive dysfunction[3]. Moreover, many studies indicate obesity during midlife increases the risk of dementias such as Alzheimer's disease later in life[4,5]. The possible mechanisms that obesity might be impacting cognitive function are early changes in neuro-chemical composition related to neuro-inflammation[6]. Previous studies reported that higher plasma levels of interleukin (IL)-12 and 6 are linked to reduced speed in processing information and a faster rate of cognitive decline[7,8].

Although evidence suggests the relationship between obesity and T2DM, an additive effect of obesity on type 2 diabetes on cognitive impairment has not been investigated. This is critical because obesity is a modifiable risk factor for DM. As an initial step to address the association between a number of cardiovascular risk factors and cognitive function, we performed a cross-sectional study involving middle-aged lean diabetes, diabetes with obesity and healthy sedentary individuals.

The mechanisms underpinning diabetes and obesity with cognitive impairment have been the subject of debate, but may include vascular degenerative process. It has been reported that individuals with either diabetes[9,10] or obesity[11,12] have an increase in stiffness of central elastic arteries. Although the associations between diabetes and arterial stiffness, and obesity and arterial stiffness have been explored in previous studies, the degree to which obesity mediates the association between diabetes and arterial stiffness has not been determined.

Moreover, evidence showed that central elastic arterial stiffness is associated with brain aging and cognitive decline[13,14]. Indeed, obese diabetes patients may be prone to have higher level of central arterial stiffness than lean diabetes; it is plausible to speculate that arterial stiffness mediated an association between cognitive function and number of cardiovascular disease risk factors.

To the best of our knowledge, the contribution of arterial stiffness to the mechanisms of cognitive impairment observed in obese type 2 DM patients has not been published. Thus, the overall goal of this study was to investigate whether arterial stiffness and cognitive function in diabetes with obesity are different from those with normal body mass index (BMI) diabetes and age-matched healthy participants.

Materials and methods

Subjects

We studied 22 sedentary healthy, 20 diabetes with normal BMI and 18 diabetes with obese subjects (age range 40-65 years). All of the diabetes subjects were diagnosed of type 2 diabetes according to the American Diabetes Association Guidelines[15], or current treatment with glucose lowering drugs. Diabetes with obesity had BMI ≥ 25 kg/m^2. Exclusion criteria include smoking, documented coronary and cerebrovascular disease, chronic kidney disease, current insulin therapy, non-cardiovascular medications interfering with vascular function including hormonal therapy, steroidal and non-steroidal anti-inflammatory drugs and resting systolic blood pressure greater than 160 mmHg and/or resting diastolic blood pressure greater than 110 mmHg. Control subjects were healthy, non-smoking, non-obese, and free of overt cardiovascular disease or diabetes assessed by screening health

questionnaire, blood pressure measurement and blood chemistry. All subjects gave written informed consent for their participation in the study which was approved by the Mahidol University Ethics Committee.

Before they were tested, subjects had abstained from food, alcohol, and caffeine for at least 4 hours (overnight 12-hour fast for metabolic risk factors). Premenopausal women were tested during the early follicular phase of the menstrual cycle. Each participant rested supine for 15 min in a quiet dimly lit, temperature-controlled laboratory room (24 ± 2 °C).

Anthropometric measurements

Body weight was determined on two occasions using an electronic scale, with the subject wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Diabetic participants who have a value higher of 25 kg/m^2 would be considered overweight and obesity. Waist circumference was measured as following: the upper border of the iliac crests will be located, and the tape would be wrapped around above this point, ensuring that it was adjusted without compressing the skin.

Pulse wave velocity (PWV) measurement

Heart rate (HR), blood pressure (BP), and pulse wave velocity (PWV) were measured by the automatic vascular screening device (VP-1000; Colin Corp. CO., Ltd, Komaki, Japan). Subjects were instructed to rest quietly for 10 minutes in the supine position. Applanation tonometry was used to detect pressure waveforms. Heart rate, bilateral brachial and ankle blood pressures, carotid and femoral pulse waves were measured. Central and peripheral PWV were automatically calculated as the ratio of the surfaced distance between the two recording sites and wave transit time[16]. Ankle brachial index (ABI) was calculated by the ratio of the ankle systolic pressure and brachial systolic pressure. The measurement was repeated three times at 2 min intervals. Average HR, BP, PWV and ABI were calculated and used for data analyses.

Cognitive testing

Mild cognitive impairment (MCI) was assessed using the Montreal Cognitive Assessment (MOCA) which is a paper-and-pencil format test. Scores range from 0 to 30. An initial validation study concluded that score below 25 is likely to indicate MCI. Previous study reported that the sensitivity and specificity of MOCA were 67% and 93% respectively in the T2DM with mean age of 59.9 ± 7.1 years[17].

Blood chemistry

Blood samples were obtained from an antecubital vein after an overnight fast at local diabetes outpatient clinic. Metabolic risk factors for cardiovascular disease including glucose, cholesterol, and triglycerides were analyzed from plasma samples.

Statistical analyses

Diabetic, diabetic with obesity patients and age-matched sedentary controls were compared using one-way ANOVA. Pearson correlation coefficients were used to examine the association between arterial stiffness, cognitive function and cardiovascular risk factors. A significance level of p-value < 0.05 was used to determine statistical significance.

Results

As presented in Table 1, there were no group differences in age and height. BMI and waist circumference were higher *(p*-value < 0.05*)* in diabetes with obesity group than in sedentary controls and diabetic patients. Sedentary control had significantly lower fasting plasma glucose, total cholesterol, low-density lipoprotein cholesterol and triglyceride concentrations than diabetes with obesity. Total cholesterol concentrations were significant higher in diabetic patients than in sedentary controls.

There was no significant difference among groups in heart rate at rest. Brachial systolic blood pressure, mean blood pressure, diastolic blood pressure and pulse pressure were higher *(p*-value < 0.05*)* in diabetes with obesity and diabetic patients than in sedentary controls.

Table 1 Selected subject characteristics

Variable	Sedentary (n = 22)	Diabetes (n = 20)	Diabetes with obesity (n = 18)
Men / women	2 / 20	3 / 17	4 / 14
Age (years)	54 ± 7	57 ± 7	54 ± 6
Height (cm)	157 ± 6	154 ± 8	160 ± 7
Body mass (kg)	53.6 ± 6.6	56.4 ± 7.2	77.2 ± 14.5 * ϒ
Body mass index (kg/m^2)	21.7 ± 2.4	23.3 ± 1.5	29.7 ± 4.3 * ϒ
Waist circumference (cm)	74 ± 5	79 ± 6	96 ± 8 * ϒ
Plasma glucose (mg/dl)	89 ± 12	131 ± 29*	136 ± 30 * ϒ
Total cholesterol (mg/dl)	193 ± 29	214 ± 32	243 ± 25 *
Low-density lipoprotein cholesterol (mg/dl)	132 ± 48	145 ± 30	152 ± 35 *
High-density lipoprotein cholesterol (mg/dl)	47 ± 5	44 ± 8	45 ± 8
Triglyceride (mg/dl)	155 ± 72	147 ± 56	167 ± 67 * ϒ
Heart rate (beats/min)	60 ± 2	60 ± 3	68 ± 2
Systolic blood pressure (mmHg)	118 ± 9	136 ± 11*	135 ± 11*
Mean blood pressure (mmHg)	92 ± 10	104 ± 6*	107 ± 14*
Diastolic blood pressure (mmHg)	71 ± 11	81 ± 6*	87 ± 9*
Pulse pressure (mmHg)	45 ± 8	56 ± 10*	57 ± 12*

Note: Values are means ± SD. * *p*-value < 0.05 versus sedentary, ϒ *p*-value < 0.05 versus diabetes.

Central artery stiffness measured by carotid-femoral pulse wave velocity and brachial-ankle pulse wave velocity was higher (*p*-value < 0.05) in diabetes with obesity and diabetic patients than in sedentary controls (Table 2). Carotid-femoral pulse wave velocity and brachial-ankle pulse wave velocity of diabetes with obesity tended to be higher than in diabetic patients but this did not achieve statistical significance (*p*-value = 0.08 and *p*-value = 0.07 respectively).

Unlike measurements of central artery stiffness, measurements of peripheral artery stiffness, femoral-ankle pulse wave velocity, were not different among 3 groups. Ankle brachial index, screening variable for peripheral arterial disease, was higher (*p*-value < 0.05) in diabetes with obesity than in diabetic patients and sedentary controls.

Table 2 Pulse wave velocity (PWV) and ankle brachial index (ABI)

Variable	Sedentary (n = 22)	Diabetes (n = 20)	Diabetes with obesity (n = 18)
Carotid-femoral PWV (cm/sec)	825 ± 124	1012 ± 90*	1083 ± 105*
Brachial-ankle PWV (cm/sec)	1329 ± 112	1692 ± 77*	1760 ± 109*
Femoral-ankle PWV (cm/sec)	1083 ± 137	1157 ± 171	1162 ± 105
Ankle-brachial index	1.08 ± 0.07	1.13 ± 0.08	1.18 ± 0.06*

Note: Values are means ± SD. *p-value < *0.05* versus sedentary. PWV, pulse wave velocity. Cognitive function measured by Montreal Cognitive Assessment (MOCA) score was higher (p-value < *0.05*) in diabetes with obesity than in diabetic patients and sedentary controls (Figure 1).

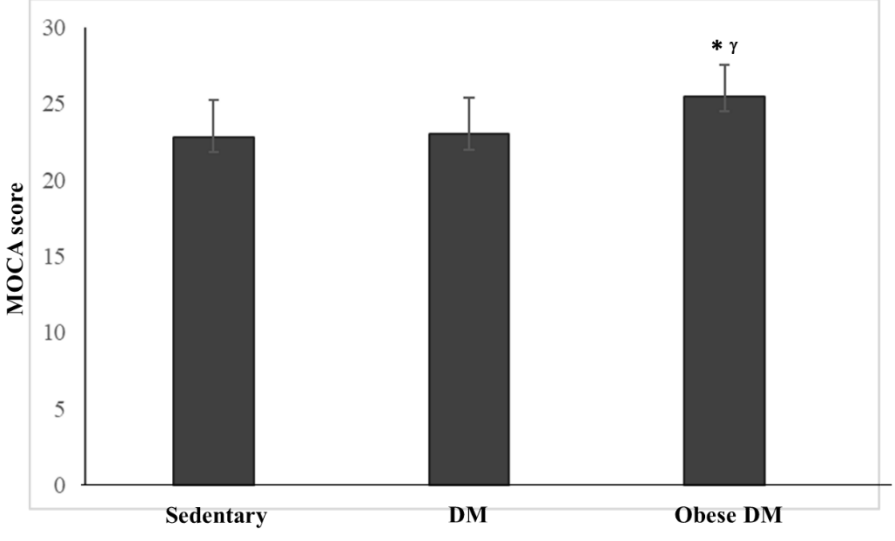

Figure 1 Montreal Cognitive Assessment (MOCA score)
Note: DM, diabetes; Values are means ± SD. *p-value < 0.05 versus sedentary; ᵞp-value < 0.05 versus diabetes.

Brachial-ankle pulse wave velocity (Figure 2) and waist circumference (Figure 3) were positively associated with MOCA score in the pooled population (r = 0.33, p-value < 0.05 and r = 0.35, p-value < 0.05 respectively). However, there were no relationship between BMI and MoCA score.

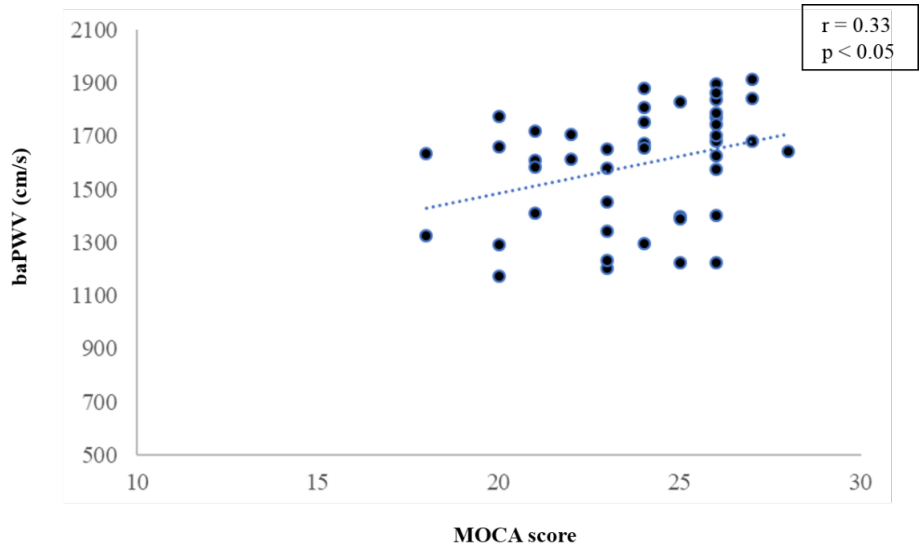

Figure 2 Association between brachial-ankle pulse wave velocity and Montreal Cognitive Assessment (MOCA) score

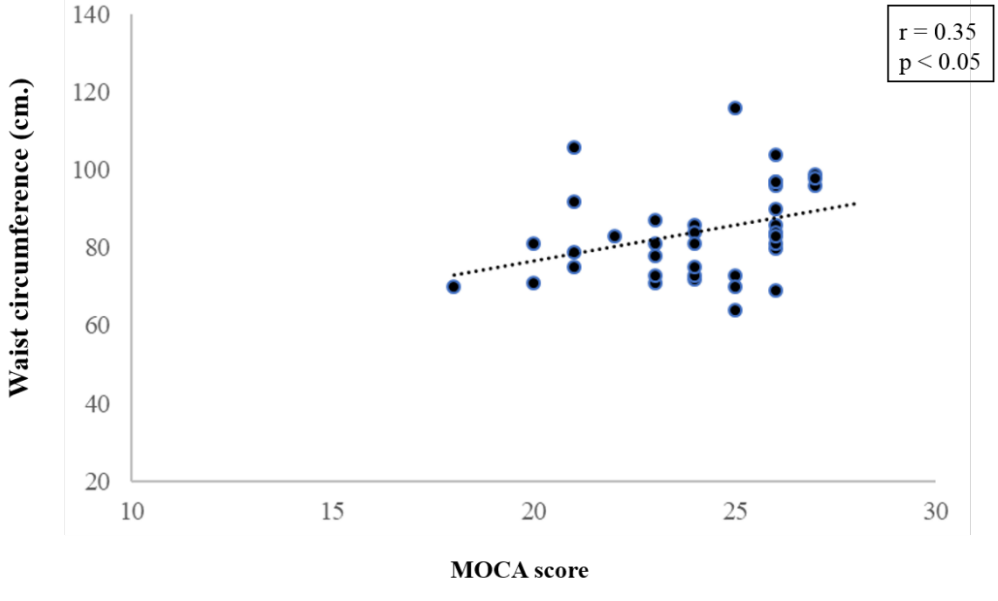

Figure 3 Association between waist circumference and Montreal Cognitive Assessment (MOCA) score

Discussion

This is the first study, to our knowledge, to determine whether diabetes with obesity exhibited higher cognitive function impairment than diabetes with normal weight. We found that diabetes with obesity group had higher MOCA score than diabetes with normal weight. This finding is in contrast with our hypotheses. The obese diabetic patients in our study showed the better cognitive ability than normal weight diabetes.

In the present study, middle-aged diabetic patients showed higher level of central artery stiffness than sedentary controls. This finding is in agreement with previous cross-sectional studies which reported high level of arterial stiffness in diabetic patients[18,19]. We first demonstrated

in the present study that carotid-femoral pulse wave velocity and brachial-ankle pulse wave velocity of diabetes with obesity tended to be higher than in diabetic patients (*p*-value = 0.07). Thus, obesity tended to show the coincidence effects with diabetes on central arterial stiffness level. In contrast with the central arteries, a lack of influence of high blood sugar and high BMI on peripheral arterial stiffness is attributed to the fact that peripheral arteries do not exhibit the same extent of pulsatile changes in diameter compared with central arteries.

It remains unclear why diabetic patients have high risk of cognitive impairment. The possible mechanism is that stiffening of elastic arteries decreases their buffering ability and increases the velocity of the propagating pressure wave which causes facilitation of pulsatile hemodynamic energy into the delicate microcirculation in the brain[20]. Recent evidence showed that elevated arterial stiffness and pulse pressure are associated with lower subcortical perfusion because of increased microvascular resistance[21]. Importantly, cerebral hypo-perfusion which leads to hypoxia is increasingly recognized as elevating the risk of dementia[22,23]. Nevertheless, it should be noted that not all individuals that present with these early cerebral abnormalities progress to dementia. In our study, the higher level of arterial stiffness in diabetic obesity group was not related to the level of cognitive impairment.

Different cardiovascular risk factors including diabetes, hypertension, and obesity were previously reported to convey a similar risk of dementia[24]. Imaging studies also showed similar cerebral changes across vascular risk factors, in particular more accentuated global atrophy and white matter hyperintensities and an increased occurrence of infarcts, although the magnitude of the effects may differ across factors[25]. The most consistent association of cardiovascular risk factors with cognitive decrements is founded for diabetes and hypertension. The previous study reported that higher pulse pressure is an important predictor of MCI[26]. Moreover, the results from previous studies for effects of obesity on cognitive function are less consistent[27].

The present study showed that the obese diabetic patients have better cognitive ability, measured by MOCA score than normal weight diabetes. However, the score differences among 3 group were small. To the best of our knowledge, there were no report about minimal clinical important difference (MCID) of the MOCA score in diabetes. Therefore, the interpretation of our results may be not generalized to other population.

The literature related to the effects of obesity on cognitive function emerged conflicting results. Some authors found the relationship between obesity as a risk of cognitive impairment[3,4]. In contrast, some evidence, which is along with our study, reported that higher body mass index (BMI) was associated with better cognitive performance[28,29]. A population-based study in 500 participants reported that the Mini-mental state examination (MMSE) score was significantly higher and the clock drawing task (CLOX) and Trail Making Test (TMT) Part A performances were better in subjects in the highest BMI (> 29.4 kg/m^2) and higher waist to hip ratio[30]. The possible pathophysiological explanation was that these subjects have greater sympathetic activation. Leptin and norarenalin level, a sympathetic nerve stimulator, is a potential cognitive enhancer[31,32].

The main limitation of our work is the cross-sectional design which did not directly determine the cause-effect relationship of cognitive function and arterial stiffness in obese diabetes patients. Moreover, magnetic resonance imaging of brain assessing white matter lesion burden has not been performed. Another potential limitation is that it was performed in a low-income bracket semi-rural area, and low statistical relationship between outcome variables level, making results not directly generalized to the population in different context.

However, some strengths merit to be emphasized. The participants are younger patients (< 65 years) and less likely to have cognitive impairment due to neurodegenerative disorder. Therefore, the other possible mechanisms of the impairment in cognitive functions in these middle-aged patients should be considered for health prevention issue.

Conclusion

In summary, the middle-aged diabetic patients showed higher level of central artery stiffness than sedentary controls. However, the arterial stiffness did not reach the statistically significant difference between T2DM with normal BMI and obese T2DM. The obese diabetic patients showed the better cognitive level measured by MOCA test than the normal BMI diabetes.

Take home messages

The results of this study showed that obese diabetes patients have higher level of central arterial stiffness than lean diabetes. The arterial stiffness and obesity are modifiable risk factors; therefore, the exercise intervention to improve vascular function and body mass index is critical to prevent vascular dysfunction in diabetes individual. Nevertheless, the population in all groups of our study showed cognitive impairment in their middle-aged. Therefore, the intervention to prevent future dementia should be considered.

Conflicts of interest

The authors declare no conflict of interest.

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Toxicity screening to human peripheral blood mononuclear cells and Wistar rats by *Crocodylus siamensis* liver extracts

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KEYWORDS
Crocodylus siamensis; Liver; Immunomodulatory; Byproducts.

ABSTRACT
Crocodile organs have been used as ingredients in traditional medicine in Asia for prevention and treatment of diseases. This study, *Crocodylus siamensis* liver was extracted to evaluate cytotoxicity and immune responses in Wistar rats. The soluble proteins part of C. siamensis' liver was prepared. In vitro cytotoxicity was assayed in human peripheral blood mononuclear cells (hPBMCs). In vivo chronic toxicity test was performed on Wistar rats with single dose. Body weights, serum biochemical parameters, serum cytokines and the histopathology were observed. The 50% cytotoxic concentration (CC50) of Crude Liver crocodile Extract (CLE) is 28.41 mg/ml. Oral administration showed no toxicity and no adverse effects for chronic period. Serum cytokines did not differ significantly between Th1 and Th2. We found the CC50 of the CLE in hPBMCs. No toxicity was observed in vivo at the study concentration, with the pro-inflammatory cytokines skewed. Since the using of traditional medicine as an alternative therapy is becoming more widely available, this study is an important beginning step to determine the toxicity of bioactive substances which found non-toxic to cells and animal model. For further study, the effectiveness of a biomolecule is compulsorily to identify. To research on their property and develop to supplement products.

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Introduction

Animal parts, including those from snakes, lizards, turtles and crocodiles, are used in traditional medicine. They can gain greater value in combination with modern and herbal drugs[1]. Crocodiles have been used in Chinese traditional medicine regimens for more than 5,000 years. *Crocodylus niloticus* blood help stabilize osmotic pressure in pregnant women[2]. Crocodile oil has been reported to heal burning skin and reduce scar formation in rats[3], which may be from its anti-inflammatory and antibacterial activities[4]. Crocodile egg protein extract combined with ginseng (*Ganoderma lucidum)* boosts the immune response[5]. The total soluble protein extracted from crocodile livers may be available to use as a supplement for the treatment of ailments.

Crocodile farms have been a growing business for meat and skin production in Thailand. Other crocodile parts, such as the bones and internal organs, including the liver, are byproducts. The liver plays a role in the synthesis of most proteins in the body and in the synthesis of detoxification enzymes. The complex functions of the liver are well described in both Western and Chinese resources[6]. However, like others natural compound, the toxicity concentration and their properties are important to be observed. In the present study, the *in vitro* and *in vivo* cellular toxicity and immune responses by Thai crocodile (*C. siamensis*) liver extract (CLE) were investigated, which may provide information concerning further applications in medicine and raise the economic value of the crocodile liver byproduct.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO) (PubChem CID:679) was purchased from Merck KGaA, Darmstadt, Germany. Penicillin (PubChem CID:5904), streptomycin (PubChem CID:19649), bovine serum albumin (BSA), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, sterile phosphate-buffered saline (PBS) pH 7.4, and trypan blue (PubChem CID:101417452) were purchased from Gibco (Grand Island, NY, USA). Ficoll-Paque™ PLUS was obtained from GE Healthcare (Uppsala, Sweden). Sterile normal saline solution was obtained from Klean & Kare™ Thailand. 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (PubChem CID:64965) was provided by Sigma Aldrich (USA).

Crocodile liver collection and extraction

Crocodile livers were obtained from Sriracha Moda Farm, Chonburi, Thailand. Two-year-old C. siamensis were harvested under a standard process. Their livers were immediately kept with standard precautions at -80 ºC until extraction.

The extraction procedure was modified from Pegg et al. (1982)[7]. Briefly, 1 kg of crocodile liver was chopped and homogenized with cold 0.9% sodium chloride at a ratio of 1:1 (w/v) using an aseptic technique. A separation step was completed by centrifugation at 12,000 × g for 40 min at 4 °C. The supernatant was collected, and the centrifugation was repeated. The clear supernatant was then removed and passed through a 0.2 µM syringe filter (PALL Life Sciences Acrodisc®, New York). This CLE was used for further experiments.

The Biuret method, modified from Nigam et al. (2007), was set up for measuring the protein present in the CLE[8]. Bovine serum albumin (BSA) was used for the standard curve construction. The CLE was mixed with 0.75 mM cupric sulfate and 94 mM sodium hydroxide in Folin-Ciocalteu's reagent. The copper (II) ions then formed a complex with the peptides, which became a violet color in the alkaline solution, and the absorbance at 540 nm was recorded and analyzed.

Human peripheral blood mononuclear cell (hPBMC) separation

The hPBMCs, separated from a healthy donor (blood donation and transfusion unit, Srinagarind Hospital, Khon Kaen, Thailand), were used as the normal primary immune cells. The study design of donor blood was approved under the office of the Khon Kaen University Ethics Committee for Human Research (HE611553). The separation procedure was modified from Heo et al. (2009)[9]. The buffy coat from 5 healthy donors was diluted with sterile 1X PBS at pH 7.4 at

a ratio of 1:3. The diluted blood was overlaid on Ficoll-Paque at a density of 1.077 g/ml, followed by centrifugation at 400× g for 40 min at 22 °C. The hPBMCs, which appeared as a cloudy layer in the middle of a centrifuge tube, were then removed and kept at -80 ºC until the next experiment.

In vitro cytotoxicity test of the CLE

The cytotoxicity of the CLE used a tetrazolium colorimetric assay modified from Twentyman and Luscombe (1987)[10]. The hPBMCs (2×10^5 cells per well), which represented immune cells, were cultured in RPMI-1640 medium containing 1% penicillin and streptomycin and 10% FBS and maintained at 37°C and 5% CO_2 under humidified conditions. The various protein concentrations of CLE, prepared by a ten-fold serial dilution with culture media, were directly added to the cell culture plates and incubated for 24 hours. The medium was then removed, and 200 µl of 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was added followed by a 3-hour incubation under the same conditions. Then, the formazan crystals, which represent viable cells, were dissolved in DMSO and the absorbance was measured by a spectrophotometer (Tecan, Tecan Trading AG, Switzerland) at 550 nm.

In vivo chronic toxicity test of CLE in Wistar rats

Twenty-six to twenty-eight weeks old male and female Wistar rats were provided by the National Laboratory Animal Centre, Mahidol University, Thailand. The rats were maintained at Northeast Laboratory Animal Centre, Khon Kaen University, Khon Kaen, Thailand. The animal experimental design was investigated in accordance with OECD No. 478 guidelines and was approved by the Khon Kaen University Ethics Committee (NELAC 21/2557). All rats were acclimated for one week under standard conditions (12 h light/dark cycle at 20-25°C and 50%–60% humidity with a commercial pellet diet and water with added chlorine at a concentration of 3-4 ppm). Next, the rats were randomly separated into four groups (5 per group) as follows: group 1) male rats receiving 3.0 mg of CLE/kg body weight; group 2) male rats receiving 1.0 ml of sterile normal saline/kg body weight; group 3) female rats receiving 3.0 mg of CLE/kg body weight; and group 4) female rats receiving 1.0 ml of sterile normal saline/kg body weight. All groups were orally given CLE or saline every other day by gavage for a chronic period (24 weeks). An animal pellet basal diet and drinking water were freely accessible. Rats were observed daily, and their body weights were measured every week. Blood was collected from the lateral tail vein every month for determination of the hematological parameters by using a Sysmex automated analyzer (Model xs-800i, Germany). Plasma biochemical parameters were determined by a Beckman Coulter automated analyzer (Model Synchron LX20pro, USA.), including a kidney function test (urea nitrogen, creatinine), liver enzymes (aspartate aminotransferase, AST; alanine aminotransferase, ALT; and alkaline phosphatase, ALP), and lipid profile (triglycerides; cholesterol; high density lipoprotein, HDL; and low density lipoprotein, LDL). After 24 weeks of the experiment, the rats were sacrificed with an anesthetic, and blood was drawn from the aorta. The internal organs, including the lungs, stomachs, intestines, kidneys and livers, were removed and fixed with 10% formaldehyde solution for evaluation of pathologic appearance by hematoxylin and eosin (H&E) staining.

Plasma cytokines assay

At the end of the experiment, the rats were sacrificed by euthanasia, and their whole blood was quickly drawn by aortic puncture and kept in a tri-potassium-ethylenediaminetetraacetic acid (3K-EDTA) tube. The plasma was separated by centrifugation at 3,500 rpm for 10 min and kept at -80°C. Plasma cytokine levels (IFN-γ, IL-2, IL-4, IL-10) and the Th1:Th2 ratio after 24 weeks of oral CLE administration were then analyzed by commercial ELISA kits (Bio-Plex assay®, Bio-Rad, USA).

Tissue processing and histological scoring

Rat kidney and liver tissues were fixed with 10% buffered formalin, followed by removal of the fixative in distilled water. Dehydration was performed by a tissue processor with serial dilutions of alcohol (70%, 90%, and 100%), clearing

of the samples with xylene and impregnating the tissues with molten paraffin wax. The tissues were embedded, sectioned (4 μm thickness) and stained with hematoxylin and eosin (H&E)[11].

The histological features of the livers and kidneys were evaluated for histotoxicity with broad categories. The histological scoring definitions are shown in Table 1, which was modified from[12,13] and (Table S1), which was modified from[14]. The tissues were scored under a light microscope in 5 random fields per sample (N = 3) by two independent pathologists.

Table 1 Histological scoring definitions of the liver

Item	Definition (% of tissue affected)	Grade
Steatosis	< 5%	0
	5-33%	1
	33-66%	2
	66-100%	3
Hepatocellular injury	None	0
Ballooning cells	Few ballooning cells	1
	Many ballooning cells/prominent ballooning	2
Acidophil bodies	None to rare	0
	Many	1
Fibrosis	Mild, perisinusoidal/periportal/zone 3	1
	Moderate, perisinusoidal/periportal/zone 3	2
	Bridging fibrosis	3
	Cirrhosis	4
Miscellaneous features	None to rare	0
	Many	1

Statistical analysis

The cytotoxicity data were compared by the nonparametric t-test. Animal experiments were analyzed by the nonparametric one-way, Mann-Whitney *U* Test (Graph Pad Prism software version 5, GraphPad Software, CA, USA) for comparing groups and cell types. Statistically significant differences were judged at a *p*-value < 0.05.

Results

Cytotoxicity of CLE on human peripheral blood mononuclear cells (hPBMCs)

To examine the toxicity of CLE against hPBMCs, the cells were extracted from normal healthy donors. The hPBMCs represent normal immune cells and were treated with CLE at various concentrations: 0, 0.006, 0.01, 0.02, 0.1, 0.5, 1.0, 1.5, 3.0, and 6.0 mg/ml for 24 h. The concentration of 0 mg/ml was the control condition (medium alone), which represents 100% cell viability. The percent cell viability was measured with the MTT assay. CLE at a concentration of 28.41 mg/ml showed 50% toxicity to hPBMCs (Figure 1).

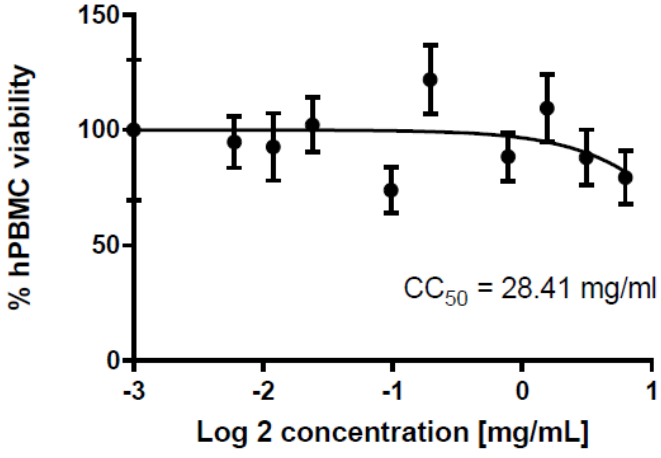

Figure 1 CC_{50} of the CLE test on hPBMCs. Data from triplicate experiments were repeated 5 times. Each bar shows the mean ± SEM.

Chronic toxicity of CLE to Wistar rats

CLE, at a concentration of 3.0 mg/kg rat body weight, was fed to rats for 24 weeks. All rats survived until the end of the experiment with no morbidity and no behavioral changes. Only one female rat, which was treated with CLE, showed illness and lethargy during the last week of the experiment. The body weight data were recorded every week (Figure 2A, B). The mean body weight of the CLE-treated group showed no significant difference compared with the control group (p-value > 0.05). This result implied that CLE had no effect on the growth of rats.

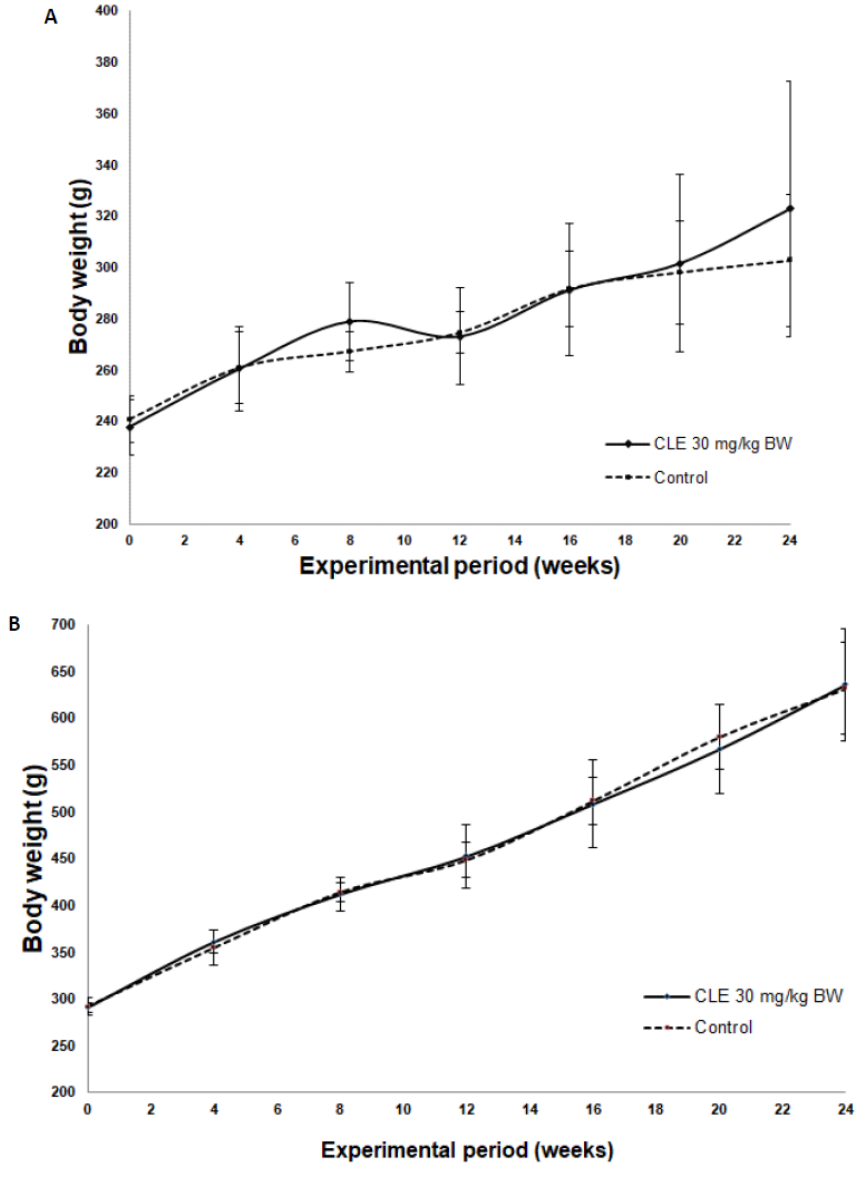

Figure 2 Effect of the oral treatment of CLE to (A) female and (B) male rat body weights (N = 5 per group). Each dot represents the result of body weight measurements at weeks 0, 4, 8, 12, 16, 20, and 24.

Clinical chemical and hematological parameters

The effects of CLE on the clinical chemical and hematological parameters in normal male and female rats were assessed after 24 weeks of the experiment. Compared with the control group, there was no statistically significant change in any of the blood hematological or clinical chemistry parameters (Tables S2, S3) of both the male and female groups treated with 3.0 mg/ml CLE. The rat intestines showed no pathological appearance (data not shown).

Plasma cytokines and Th1: Th2 cytokine balance

Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) plasma cytokines were measured in both female and male rats to evaluate the effect of CLE or their

metabolites on cytokine patterns, as shown in Table S4. The levels of IFN-γ, IL-2, IL-4 and IL-10 independent analysis was not significantly different between the control and CLE group in both male and female. The Th1:Th2 ratio was calculated to observe the type of immune response. Figure 3A shows that the Th1:Th2 ratio in female rats treated with CLE showed significant differences: IFN-γ/IL-4 (*p*-value = 0.008) and IFN-γ/IL-10 (*p*-value = 0.019) compared to the control group. The result of male group including IFN-γ/IL-4 (*p*-value = 0.0008), IFN-γ/IL-10 (*p*-value = 0.016), IL-2/IL-4 (*p*-value = 0.004), and IL-2/IL-10 (*p*-value = 0.001), are shown in Figure 3B.

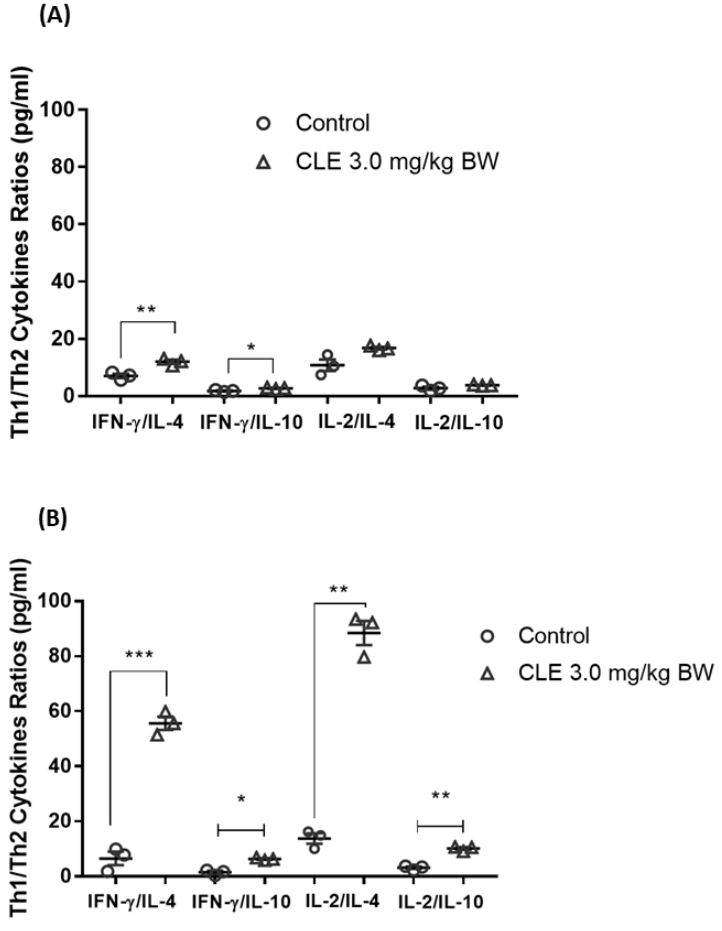

Figure 3 Th1: Th2 cytokine balance pattern of the rats after 24 weeks of CLE oral administration (3.0 mg/kg body weight). INF-γ/IL-4, INF-γ/IL-10, IL-2/IL-4, IL-2/IL-10 ratios were plot; (A) female (B) male. The mean ± SEM is displayed on each bar graph. $^{*}p$-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001.

Histological scores from CLE chronic oral administration to rats

After termination of 24 weeks of chronic oral administration, the internal organs such as the lungs, stomachs, intestines, kidneys, and livers were observed macroscopically for abnormalities of the prepared sections. There were no significant abnormalities found in the organ and tissue sections from the lungs, stomachs, intestines, and kidneys between the control group and the CLE 3.0 mg/kg BW treatment groups (data not shown), both male and female. However, the livers from both the male control and male CLE-treated groups and the female CLE-treated group showed

lipid droplets around the hepatic portal vein, as shown in figure 4. The histological scores of the kidneys were not affected (≤ 1) in all groups, and there was no significant difference between the groups in terms of gender control, as detailed in Table S5. The liver examinations of CLE-treated male rats group scored indicated that significantly higher the hepatocellular injury concerning the number of ballooning cells than the control male rats and showed mild steatosis (> 1) that was not significant, detail in Table S6.

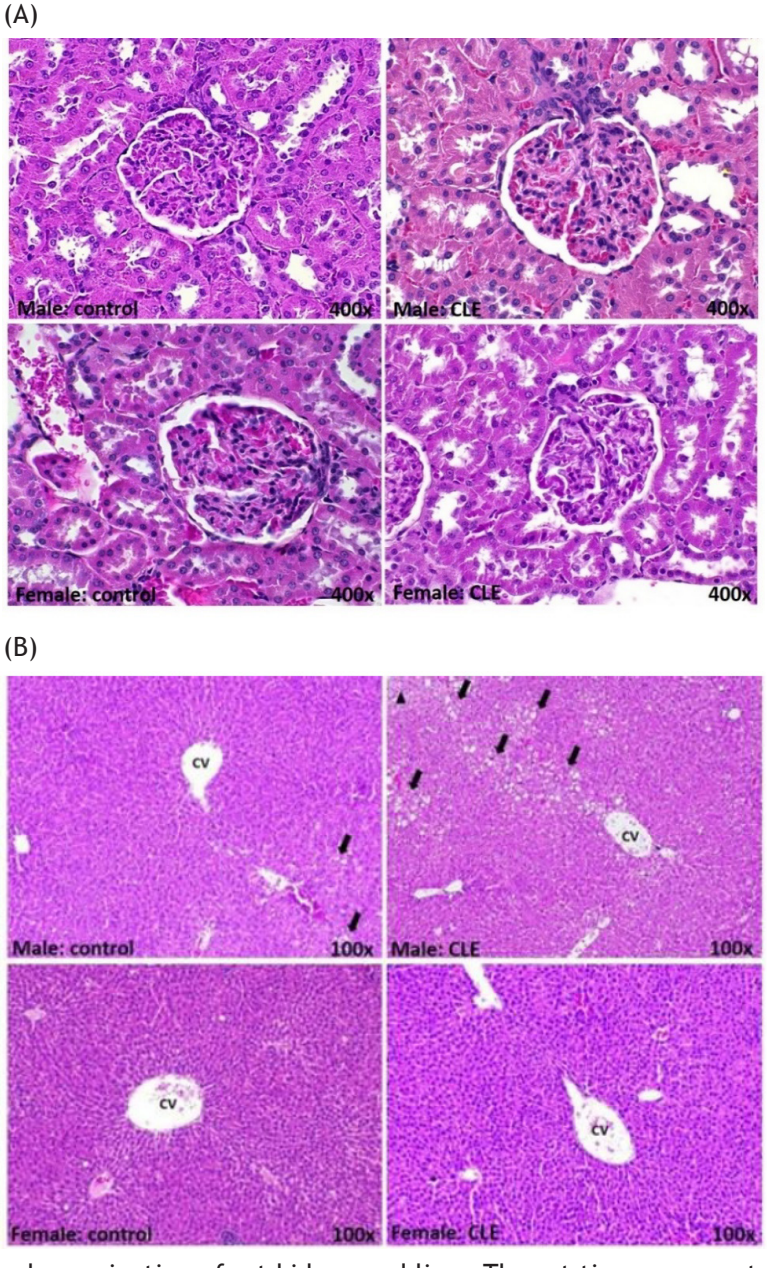

Figure 4 Histological examination of rat kidney and liver. The rat tissues were stained with H&E and observed under light-microscope (A). The kidney tissues represent with glomerulus and renal tubules each group with 400x magnification (B). The liver examinations represent hepatic structure including central vein (CV), steatosis (arrow) and ballooning cells (head arrow) with 100x magnification.

Discussion

Crocodile liver remains popular as a health-supportive bioactive ingredient in Asia and Africa traditional medicine[15]. Moreover, the farm industry of this species is seen as their internal organs to be a byproduct. The utilization of crocodile liver gives two important outcomes. The utilization of crocodile liver gives two important outcomes. First, to reduce environmental problems from the agricultural industry. Second, it is necessary to clarify the toxicity concentration of natural extracted resources for edibility safety. The overall parameters affected by CLE are determined in this study including body weight, serum biochemical, hematological, cytokines secretion, and histopathological appearance. The *in vitro* cytotoxicity test was used initially to examine the harmless concentration of CLE. The hPBMCs with which the primary cells were mixed is advantageous to use since they reflect lymphocyte, T cells, and monocyte responses. An example of peptide immunomodulatory activity was reviewed by Gauthier et al. (2006), which showed proliferation, cytokine expression, antibody production, and immune cell function[16]. In the present study, the 50 percent cytotoxicity concentration is 28.41 mg/ml of CLE. With little cyto-toxicological effect, 0.1 of CC_{50}, (3.0 mg/kg body weight) was selected as safely edible concentration for chronic period oral administration in Wistar rat. We tested the *in vivo* toxicity under OECD guidelines. A severe toxicological effect was not found, with no ruffled fur or mortality until the end of the experiment. Rat body weight gain did not decrease, indicating that the compound CLE is well-tolerated and does not produce mortality or any adverse effects in both male and female rats, which mean CLE does not risk to normal growth. According to the baseline normal range of blood parameters, the observed results are well defined in their hematological and clinical chemistry parameters[17]. No abnormal hematological parameters were found. Liver enzymes, including AST, ALT and ALP, and lipid profiles including cholesterol, triglycerides, and LDL, increased in the male CLE-treated group. In the female group, there was an increase in ALP and the lipid profile, including cholesterol, triglycerides, and LDL.

Histopathological observations focused on the kidneys and livers and reflected the toxicity of CLE with metabolite formation after long-term administration. The hepatic toxicity scoring system was modified following the studies of Zheng et al. (2005) and Kleiner et al. (2005)[13,14]. In the present study, fatty droplets around the portal vein were observed to be related to the increasing lipid profiles, while changes in other organs were not found. A fatty liver is the primary toxic effect that is mainly caused by liver metabolism or detoxification. The increase of serum lipids by more than 1 S.D. is associated with decreasing infectious diseases such as bacteremia, nervous system infection, and miscellaneous viral infections from a cohort study in both men and women. During inflammation, cytokines may alter lipid metabolism and induce lipoprotein-binding endotoxins, resulting in interruption of the cytotoxic effects[18]. Inflammatory activation and toll-like receptor activation can be induced by cholesterol accumulation by macrophages. Peripheral blood cholesterol increases resulting from decreases in macrophage cholesterol efflux probably followed the amplification of TLR signaling in macrophage cells via the ABCA1 receptor[19]. The long-term oral administration of CLE found that cholesterol, triglycerides, and LDL might be relevant to reverse the cholesterol transport pathway.

Plasma cytokines may reflect systemic activation by CLE and its metabolites of Wistar rats. We expect to see the balance between proinflammatory and anti-inflammatory cytokines in rats treated with CLE. Interestingly, we found Th1 skewed. Th1 is dominant in several conditions, including viral or intracellular pathogen infection, the fighting of cancer cells and stimulation delayed-type hypersensitivity skin reactions, whereas Th2 is dominant when the body is exposed to extracellular pathogens. Under normal conditions, the Th1/Th2 ratio balancing hypothesis is still argued[20]. Th1 dominant caused by IFN-γ and IL-2 increase, suggested that CLE might able an antigenicity to induce cellular. The ratio of IFN-γ/IL-4, IFN-γ/IL-10, IL-2/IL-4, and IL-2/IL-10 increased in male rats due to the secretion raise in Th1 cytokines, while there were higher levels of IFN-γ/IL-4 and IFN-γ/IL-10 ratio in female

rats treated with CLE. The Th1/Th2 ratio in both males and females treated with CLE exhibited Th1 dominance. This implied that CLE may affect rat cellular immunity. Taken together, CLE may induce immune cell proliferation, differentiation as Th1 was increased in the U-937 cell line (data not shown). We suggest further investigations of cytokine mediation and other immune functions with varies dose of CLE.

Conclusion

Our results are the first toxicity data from CLE. There were 40 grams of CLE powder from one kilogram of crocodile liver (wet weight) in the extraction. The *in vitro* and *in vivo* safety assessment results are related to the noncellular toxic effects in normal cells and the absence of adverse effects in rats. The cytokine pattern exhibits balancing and skewing toward cellular immunity. Detection of fat droplets in hepatocytes is a reversible phase of toxicity related to an increase in the lipid profile, and fat removal is an important the next step of the application. Furthermore, the *in vivo* plasma cytokine results relate to the *in vitro* cell base assay. The safety assessment of CLE is important to determine for future applications. In a further study, we attempted to test other different immune functions in both *in vitro* and *in vivo* pathological condition.

Clinical implications

- Cytotoxicity of crude liver extract as an edible supplement is compulsory to be evaluated.
- In vivo study act for the toxicity of the extract at chronic oral administration.
- The study model might be a fundamental experiment for supplement test.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary

Table S1 Histological scoring definitions of the kidneys for glomerular injury, tubular cast formation, tubular necrosis, and interstitial inflammation

	Grading				
	0	1	2	3	4
Percent of tissue affected	No disease	1 - 25%	26 - 50%	51 - 75%	76 - 100%

Table S2 Effect of CLE oral administration (3.0 mg/kg body weight) for 24 weeks on the hematological parameters of rats[1]

Parameter	Unit	Male rats		Female rats	
		Control	CLE	Control	CLE
RBC	$\times 10^6$/µl	9.16 ± 0.34	10.08 ± 0.71	8.16 ± 1.50	9.75 ± 0.39
Hemoglobin	g/dl	16.65 ± 0.78	17.27 ± 0.55	14.65 ± 2.76	16.2 ± 0.35
Hematocrit	%	52.40 ± 0.71	50.73 ± 2.19	45.00 ± 10.75	47.60 ± 2.16
MCV	Fl	57.25 ± 1.34	48.60 ± 1.08	54.85 ± 3.04	48.84 ± 2.36
MCH	Pg	18.15 ± 0.21	16.53 ± 0.57	17.95 ± 0.07	16.62 ± 0.40
MCHC	g/dl	31.80 ± 0.99	34.07 ± 0.55	32.80 ± 1.70	34.08 ± 1.49
RDW	%	19.05 ± 0.21	22.93 ± 1.43	17.05 ± 2.19	21.44 ± 1.44
WBC	$\times 10^3$/µl	7.24 ± 2.09	7.55 ± 1.91	3.55 ± 0.65	7.29 ± 1.21
Neutrophils	%	14.75 ± 1.34	11.58 ± 1.60	17.05 ± 0.21	14.0 ± 6.06
Eosinophils	%	0.70 ± 0.00	1.64 ± 0.40	1.15 ± 0.78	2.32 ± 2.44
Lymphocytes	%	86.60 ± 7.78	83.78 ± 2.06	78.55 ± 0.78	78.5 ± 8.75
Monocytes	%	4.55 ± 0.21	2.86 ± 1.15	3.25 ± 1.77	3.72 ± 2.49
Basophils	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets	$\times 10^3$/µl	828.00 ± 77.78	1007.67 ± 247.66	761.50 ± 3.54	712.80 ± 300.03
MPV	Fl	10.50 ± 1.41	8.50 ± 0.92	9.20 ± 0.28	8.36 ± 1.14

Note: [1] N = 5 per group; Control = rats received 1 ml of sterile normal saline/100 g of body weight; Fl, Femtolitre; Pg, Picogram; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MPV, Mean platelet volume; RDW, Red blood cell distribution width; RBC, Red blood cell; WBC, White blood cell.

Table S3 Effect of CLE oral administration (3.0 mg/kg body weight) for 24 weeks on the clinical chemistry parameters of rats[1]

Parameter	Unit	Male rats		Female rats	
		Control	CLE	Control	CLE
Urea nitrogen	mg/dl	25.00 ± 3.61	24.80 ± 1.48	23.00 ± 4.30	24.60 ± 2.51
Creatinine	mg/dl	0.58 ± 0.23	0.71 ± 0.09	0.60 ± 0.15	0.72 ± 0.13
AST	U/l	105.67 ± 60.08	171.40 ± 42.51	227.40 ± 280.40	163.40 ± 38.74
ALT	U/l	57.00 ± 11.79	129.60 ± 84.18	116.80 ± 162.90	123.00 ± 68.12
Alkaline phosphatase	U/l	36.67 ± 7.37	99.60 ± 41.14	53.00 ± 29.89	116.60 ± 28.60
Triglyceride	mg/dl	126.50 ± 2.12	240.40 ± 70.61	117.80 ± 40.36	169.00 ± 46.53
Cholesterol	mg/dl	54.50 ± 19.09	79.60 ± 8.44	48.40 ± 9.42	70.40 ± 13.01
HDL	mg/dl	39.50 ± 13.44	36.60 ± 5.86	31.20 ± 4.32	35.60 ± 5.77
LDL	mg/dl	41.96 ± 10.50	98.25 ± 16.58	44.07 ± 27.81	77.47 ± 37.82

Note: [1] N = 5 per group; Control = rats receiving 1 ml of sterile normal saline/100 g body weight; AST, Aspartate transaminase; ALT, Alanine transaminase; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; U, unit; Fl, Femtolitre; Pg, Picogram; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MPV, Mean platelet volume; RDW, Red blood cell distribution width; RBC, Red blood cell; WBC, White blood cell.

Table S4 Plasma cytokine concentrations of rats after 24 weeks of CLE oral administration (3.0 mg/kg body weight)

Cytokine (pg/ml)	Male rats		*p*-value	Female rats		*p*-value
	Control	CLE		Control	CLE	
IFN-γ	97.89 ± 25.11	80.39 ± 35.36	0.703	109.70 ± 11.57	104.20 ± 18.65	0.812
IL-2	187.10 ± 17.84	1147.50 ± 52.11	0.524	168.50 ± 31.41	170.40 ± 4.86	0.957
IL4	19.06 ± 2.55	15.84 ± 5.73	0.634	17.64 ± 4.08	15.87 ± 1.36	0.721
IL-10	76.91 ± 9.41	57.83 ± 18.39	0.407	64.75 ± 9.27	46.42 ± 5.95	0.157

Note: Control = rats receiving 1 ml of sterile normal saline/100 g body weight.

Table S5 Histological scores of the kidneys

Item	Male		Female	
	Vehicle control	CLE 3.0 mg/kg BW	Vehicle control	CLE 3.0 mg/kg BW
Glomerular injury	0.10 ± 0.05	0.03 ± 0.05	0.13 ± 0.09	0.13 ± 0.00
Tubular cast formation	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tubular necrosis	0.30 ± 0.05	0.27 ± 0.09	0.27 ± 0.09	0.17 ± 0.05
Interstitial inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table S6 Histological scores of the livers

Item	Male		Female	
	Vehicle control	CLE 3.0 mg/kg BW	Vehicle control	CLE 3.0 mg/kg BW
Steatosis	0.30 ± 0.05	1.44 ± 0.63	0.23 ± 0.14	0.00 ± 0.00
Hepatocellular injury				
• Ballooning cells	0.09 ± 0.03	1.16 ± 0.41*	0.18 ± 0.06	0.00 ± 0.00
• Acidophil bodies	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Fibrosis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Miscellaneous features	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Note: * statistically significant at *p*-value < 0.05.

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Reduction of need for repeat chest x-rays caused by insufficient inspiration through enhanced patient communication

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KEYWORDS
Repeat/reject analysis; Chest x-ray examination; Inadequate/ insufficient inspiration; Geriatric patients; Patient communication.

ABSTRACT
This two-phased study first (phase 1) explored the effect of patient age and gender on the repeat rate of chest x-ray examinations due to inadequate inspiration. In phase 2, we sought to reduce this rate using an instructive video to improve patient understanding of the chest examination. In phase 1, the analysis included a total of 12,905 radiographic images from all types of examinations from a 3-month period collected retrospectively. These included 1,484 repeated images which gave a total repeat rate of 11.2%. The highest proportion of repeated images were chest examinations (29.7%). Of the chest examinations, with a repeat rate of 10.0%, inadequate inspiration was the major reason (76.3%) for repeating the exam. Our analysis of these repeated examinations showed that there was a positive association between increased repeat procedures caused by inadequate inspiration and patient age (more than 40 years). In phase 2, we designed and provided an instructive video about chest x-ray examinations. We found that its use was associated with a significant reduction in the repeat rate due to inadequate inspiration among patients aged 41-60 years. Effective communication is an important role of healthcare providers and can improve the quality of medical services.

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Introduction

Repeat or reject analysis is one of the important parts of a quality assurance program for general diagnostic radiography. Parameters monitored in the repeat analysis can include image and imaging procedure quality, skills of radiological technologists, patient radiation dose and performance of equipment[1]. Discovery of the causes or factors affecting rejection of images, allows radiology departments to make changes which improve service quality and patient dosage[2-6].

Anderson et al.[3] collected and analyzed data on rejected images in order to define the causes of repeat studies in routine work. Their total rejection rate was 12%, with the highest (in decreasing order) being examinations of the knee, then shoulder, and wrist. Though having a lower rejection rate, chest examinations made up the largest percentage (27.9%) of rejected images. Using a questionnaire, they polled 15 radiographers as to the reasons for rejection in different situations. Ten of the 15 radiographers defined poor inspiration in chest x-rays as a cause of repeat studies among elderly hearing-impaired patients. These images usually showed poor inspiration and/or non-projection of the left coronary sinus[3].

Even after the transition to digital radiography, quality control analysis of rejected images remains crucial for maintaining image quality[7]. Consequently, factors affecting the need for repeat images have been analyzed, and solutions as well as guidelines have been proposed for improving service quality[2,4,6,8]. Multiple variables, including sex, age, type of examination and projection, on the need for image retakes were analyzed. As a result, guidelines were proposed in 2016 for reducing the frequency of repeat imaging[8].

In our medical center in Thailand, repeated chest examinations made up the highest proportion (one-third) of all repeated images of any x-ray type. The main indication for retake of chest examinations was inadequate inspiration during exposure. The radiological technologists suspected that such inadequate inspiration was most common in older patients. Therefore, this study was designed to explore possible associations of patient age and gender with repeated chest examination caused by inadequate inspiration (phase 1). Since effective patient communication is important in optimizing radiological and medical care[9,10], the study also sought to reduce the need for repeat chest examinations by creating and using an instructive video (phase 2).

Materials and methods

The study was approved by the Institutional Review Board for Research Ethics on Human Subjects, Multicenter Research, Mahidol University; the date of approval was December 28, 2018.

Data collection and analysis

Data acquired by the digital imaging system in the Division of Radiological Technology, Golden Jubilee Medical Center, Salaya, Nakhon Pathom, Thailand, were retrospectively collected for 3 months in 2018 (phase 1). There were 12,905 images from all radiologic examinations. Repeated images totaled 1,484; the overall repeat rate was 11.2%. The type of examination with the highest proportion of repeated images was chest examination (29.7%). The repeated images of chest x-rays were so frequent which they made up one-third of all repeat examinations. Causes of repeating chest examinations were classified into: inadequate inspiration, positioning error, patient artifact, improper exposure, user error, or miscellaneous. Criterion for full inspiration was visibility of more than 9 posterior ribs above the diaphragm. The department's radiological technologists were responsible for accepting or rejecting images. All of them had been trained in using the same criterion and had adequate experience.

Only patients 18 years of age and older were included in the analysis of association between age and gender, and repeat imaging due to inadequate inspiration to ensure cooperation during instructional video and chest examination. The analysis used simple logistic regression to determine if there was a significant association between these variables and inadequate inspiration.

The statistical analysis was performed using PASW Statistics 18 software (version 18.0.0). The significance level was defined as *p*-value < 0.05.

Design and implementation of instructive video

After analyzing the repeat rates, a video was designed to increase patient understanding with the hope that this would reduce the need for repeat chest examinations. The instructional video consisted of patient preparation, examination procedures, and patient positioning. The importance of removing metal and radiopaque materials, and of full inspiration during radiographic exposure, were emphasized. Patients were informed that these steps would enhance image quality and diagnostic accuracy. The instructive video was displayed in the waiting room and the participants 18-years-old and older were advised (but not forced) to watch it. After launching the video, chest image data were collected for another 2 months (phase 2). The repeat rates caused by inadequate inspiration were calculated and compared to rates from data collected during the two months before launching the video.

Results

During the pre-intervention period of the study (phase 1), there were a total of 12,905 radiographs performed, among which were 4,309 chest x-rays (33.4%). Overall, 430 (10%) chest x-rays needed to be repeated (Table 1). Of the 430 repeated chest x-ray examinations, the most common cause for repeat was inadequate inspiration which accounted for 76.3% of these cases, followed by positioning error causing 19.8% of cases. For evaluating the association of age and gender with the repeat rate caused by inadequate inspiration, the patients younger than 18 years (n = 205) were excluded. The frequencies of repeated chest examinations due to inadequate inspiration stratified by age and gender are shown in Table 2. Compared with the youngest age group (18 - 40 years), the odds ratios of repeat studies among the patients aged 41-60, 61-80, and more than 80 years old were 2.31, 2.27, and 2.17 (all *p*-value < 0.001), respectively. Females and males did not differ significantly in repeat rates.

Table 1 Causes of repeat chest examinations over 3 months (phase 1)

	Number of cases	Percentage of total cases	Percentage of repeated cases
Total cases	4,309	100.0	
Repeated cases	430	10.0	100.0
Inadequate inspiration	328	7.6	76.3
Positioning error	85	2.0	19.8
User error	1	0.0	0.2
Patient artifact	5	0.1	1.2
Exposure error	2	0.0	0.5
Miscellaneous	9	0.2	2.1

Table 2 Association of patient age and gender with repeated examination due to inadequate inspiration (phase1)

Patient characteristics	Number of total images	Number of repeated images (%)	Odds ratio (95% CI)	*p*-value
Age group (years)				
18 - 40	1,231	56 (4.5)	1.00	
41 - 60	902	89 (9.9)	2.31 (1.64, 3.22)	< 0.001
61 - 80	1,451	141 (9.7)	2.27 (1.65, 3.13)	< 0.001
>80	418	39 (9.3)	2.17 (1.42, 3.33)	< 0.001
Gender				
Female	2,227	173 (7.8)	1.00	
Male	1,783	152 (8.5)	1.11 (0.88, 1.39)	0.761

In phase 2 of the study, repeat rates of chest examinations due to inadequate inspiration 2-months before and 2-months after launch of the instructive video were compared (Table 3). The overall repeat rate of chest examinations caused by inadequate inspiration was reduced from 8.4% to 7.6%. When stratified by age, the repeat rate for those 41-60 years old (compared with 18-40-year-olds) decreased significantly (*p*-value < 0.05) from 10.1% to 5.7%, while changes in other age groups were negligible.

Table 3 Comparison of repeat rates of chest examinations caused by inadequate inspiration 2-months before and 2-months after launch of instructive video (phase 2)

Age (Years)	Before using the instructive video			During use of the instructive video			*p*-value
	Number of images	Number of repeated images	Repeat Rate (%)	Number of images	Number of repeated images	Repeat Rate (%)	
Total	2,591	218	8.4	2,391	181	7.6	0.273
18 - 40	729	33	4.5	540	23	4.3	0.819
41 - 60	601	61	10.1	751	43	5.7	0.002
61 - 80	968	97	10.0	900	96	10.7	0.647
> 80	293	27	9.2	200	19	9.5	0.915

Discussion

This study explored the association of patient age and gender with the repeat rates of chest examinations due to inadequate inspiration. Patients seen in this medical center presented without any special selection or referral process, and were considered to be representative of the wider population of adults in central Thailand. Our analysis showed that the repeat rate was especially high in older patients (more than age 40). This is consistent with a previous report[8] of high rejection rates in elderly patients. Of note, patient age is an important factor in the success of patient-centered instruction[11].

This study showed that our instructive video significantly reduced the need to repeat chest x-rays due to inadequate inspiration in patients aged 41- 60 years. Rates did not change

significantly in patients older than 60 years. We experienced some limitations in cooperation from patients regarding voluntarily watching the instructive video. Patient age is known to influence the perception of health care communications[10]. Among our patients 61 years and older, the repeat rates caused by the inadequate inspiration were not reduced. This may have been due to physical constraints common to geriatric patients[12] in terms of taking a deep breath during x-ray exposure. A limitation of this study was that pathological conditions of patients were not recorded and so could not be analyzed. Such factors may explain the lack of a reduction of repeat rates in the oldest age groups.

Conclusion

In conclusion, there was a direct relationship between the frequency of repeat chest x-ray due to inadequate inspiration and patient age. When an instructive video was utilized to better inform patients about the chest examination procedure, the repeat rate caused by inadequate inspiration was reduced for patients aged 41 - 60 years, but not those older. Analyses of repeat x-rays, stratified by causes and type of examination, assist in identifying problems and focusing effective corrective actions. Effective communication and instruction are important tools to improve health care service and radiographic practices for geriatric patients.

Take home messages

Effective patient-centered communication and instruction are fundamental to the success in health care services and radiologic procedures, and thus improve the quality of patient care and satisfaction.

Conflicts of interest

The authors declare no conflict of interest.

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Factors associated with multidrug-resistant *Pseudomonas aeruginosa* infection: a hospital-based case-control study

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KEYWORDS
Multidrug resistance; *Pseudomonas aeruginosa;* Mechanical ventilator; Endotracheal tube.

ABSTRACT
This study aimed to investigate the factors associated with multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) infection. The study was a hospital-based case-control study. Data were collected from medical records of patients with MDR-PA infection (110 cases) and without MDR-PA infection (110 controls) at the Roi Et Hospital between January 1, 2015 and December 31, 2017. Data analysis used statistics, univariable analysis, and multiple logistic regressions. Statistical significance was set at p-value < 0.05. Most of the 110 cases with MDR-PA infection were male (51.82 %) and the mean age was 61.82 ± 15.67 years, while most of the 110 controls were male (56.36 %) and the mean age was 55.01 ± 16.09 years. The multivariable analysis revealed that patients aged over 60 years (OR_{Adj} = 2.06, 95%CI = 1.14-3.71), admitted for more than 7 days (OR_{Adj} = 2.43, 95%CI = 1.28-4.60), underwent procedures involving an endotracheal tube (OR_{Adj} = 2.61, 95%CI= 1.42-4.80) and a mechanical ventilator (OR_{Adj} = 3.14, 95%CI= 1.67-5.88). Therefore, the risk factors for MDR-PA infection were patients who were admitted for more than 7 days, who underwent procedures involving an endotracheal tube and a mechanical ventilator.

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Introduction

The nosocomial infections caused by multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) is a major problem worldwide. These infection pathogens can cause serious problems because they are difficult to control. High mortality has been reported in patients with MDR-PA infection, especially those with septicemia[1,2]. The cost of treatment for MDR-PA is high. A study in Spain showed that the total economic cost per admission of a patient with MDR-PA was higher than that for non- multidrug resistant strains (EUR 15,265 and EUR 4,933, respectively)[3]. The risk factors for MDR-PA infection in China were patients who underwent tracheal intubation insertion, where a carbapenem was used, and hospitalization[4]. A study in Spain showed that bronchiectasis and the number of antibiotics used were independent factors for MDR-PA infection[5]. A study in Italy reported that the risk of MDR-PA septicemia was highest in patients who had a central venous catheter, previous antibiotic therapy, and corticosteroid therapy[6]. The prevalence of nosocomial infection in Thailand was reported to be highest with *Pseudomonas aeruginosa* followed by *Klebsiella* spp., *Acinetobacter baumannii*, Methicillin-resistant *Staphylococcus aureus*, and *Enterococci* spp., respectively[7]. However, despite the high prevalence and increasing numbers of MDR-PA, reporting on the risk of MDR-PA infection among Thai populations is rare. The aim of this study was to investigate the factors associated with MDR-PA infection.

Materials and methods

A hospital-based case-control study (control: case = 1:1) was used. All variables of interest were retrieved from medical records from the period January 1, 2015 to December 31, 2017. In total, there were 110 cases of patients diagnosed with MDR-PA infection and 110 controls were selected without MDR-PA infection.

Ethics consideration

This research was approved by the Ethics Committee for Human Research at Roi Et Hospital, Thailand (reference number 034/2560).

Criteria of cases

All of the 110 cases were the first diagnosis of *MDR-PA* infection, were admitted for more than 48 hours and were older than 15 years with no previous reported bacterial infection. All cases were confirmed diagnosis by physicians following the standard guidelines from the Department of Infectious Controls based on culture and sensitivity testing results for each of the study subjects.

Criteria of controls

None of the 110 controls had MDR-PA infection, none of them had reported for others bacterial infection and were randomly selected from patients who had been admitted on the same day as the cases, having been admitted for more than 48 hours and were older than 15 years.

Variables of interests

The variables of interest were: 1) general characteristics: sex, age, education, marital status, occupational, complication, duration of admission, and ward admitted to; and 2) risk factors from exposure to medical procedures: mechanical ventilator, urinary catheterization, endotracheal tube, and surgery. All variables were categorized into two groups (No/Yes).

Laboratory testing for MDR-PA

For *P. aeruginosa* identification, the clinical specimens from patients were cultured on blood agar and MacConkey agar at 33-35 °C and incubated for 16-18 hours. On blood agar, *P. aeruginosa* colonies show as beta-hemolytic or gamma-hemolytic and show as non-lactose fermented colonies on MacConkey agar. Standard biochemical tests were used for the identification of *P. aeruginosa*. The laboratory testing process was performed in the Department of Clinical Microbiology Laboratory, Roi Et Hospital.

Susceptibility tests for *MDR-PA* were used disc diffusion. Discs test consisted of amikacin (AK, 30 µg), ceftazidime (CAZ, 30µg), imipenem (IPM, 10µg), meropenem (MEM, 10 µg), doripenem (DOR, 10 µg), piperacilin/tazobactam (TZP, 110 µg), gentamicin (GN, 10 µg), co-trimoxazole (SXT), and ciprofloxacin (CIP, 5 µg). The quality controls for the antibiotic susceptibility tests were used *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

MDR-PA was defined as P. aeruginosa resistant to more than three groups of antibiotics consisting of aminoglycoside, carbapenem, cephalosporin, beta-lactam plus beta-lactamase inhibitor, and quinolone.

Statistical analysis

The analyses were used descriptive statistics and Pearson's Chi-square test for homogeneity testing between cases and controls. Bivariate and multivaritate analyses were used logistic regression to investigate the associations among the factors of MDR-PA infection. Bivariate analyses used simple logistic regression for each variable and outcome; *p*-value tested at less than or equal to 0.25 from Wald's test were entered into the initial model of multivariate analysis using a backward elimination method. A *p*-value from Wald's test of less than 0.05 was considered significant. The final model was adjusted by sex and age and presented using the adjusted odds ratio (OR_A), 95% confident interval, and *p*-value. Statistical significance was set at *p*-value < 0.05.

Results

General characteristic of cases and controls

Of the 110 cases, 51.82% were male and 48.18% were female. The mean age was 61.82 years (SD = 15.67), 91.82% had graduated from primary school, 76.36% had a spouse, 67.27% worked in agriculture, 40.91% had been admitted to the intensive care unit, and 54.55% had been admitted for longer than 7 days. Most of the 110 control subjects were male (56.36 %), the mean age was 55.0 years (SD = 16.09), 71.82% had graduated from primary school, 60.00% had a spouse, 57.27% worked in agriculture, 45.45% had been admitted to the intensive care unit, and 61.82% had been admitted for 7 days or less (Table 1).

Table 1 General characteristic of cases and controls

Variable	Cases (n = 110)	Controls (n = 110)	*p*-value
	n(%)	n(%)	
Sex			0.499
Male	57(51.82)	62(56.36)	
Female	53(48.18)	48(43.64)	
Age (years)			0.004
≤ 60	49(44.55)	70(63.64)	
> 60	61(55.45)	40(36.36)	
Mean ± SD	61.82 ± 15.67	55.01 ± 16.09	
Min, Max	18, 87	17, 86	
Education			<0.001
Primary school	101(91.82)	79(71.82)	
High school	6(5.45)	12(10.91)	
Bachelor' s degree	3(2.73)	19(17.27)	
Marital status			0.005
Single	20(18.18)	21(19.09)	
Spouse	84(76.36)	66(60.00)	
Divorced	2(1.82)	4(3.64)	
Widowed	4(3.64)	19(17.27)	

Table 1 General characteristic of cases and controls

Variable	Cases (n = 110)	Controls (n = 110)	*p*-value
	n (%)	n (%)	
Occupational			0.162
Agriculture	74(67.27)	63(57.27)	
Business	3(2.73)	2(1.82)	
Government officer	4(3.64)	11(10.00)	
Employee	13(11.82)	21(19.09)	
Unemployed	16(14.55)	13(11.82)	
Ward admitted to			0.004
Surgical	12(10.91)	14(12.73)	
Medicine	35(31.82)	32(29.09)	
Intensive care unit	45(40.91)	50(45.45)	
Others	18(16.36)	14(12.73)	
Duration of admittance	50(45.45)	68(61.82)	0.015
≤ 7 days	60(54.55)	42(38.18)	
> 7 days			

Note: *p*-value from chi-square test.

The antibiotic susceptibility pattern of MDR-PA

The 110 case of MDR-PA were susceptible to amikacin 80.4%, ceftazidime 18.3%, ciprofloxacin 19.9%, gentamicin 21.8%, imipenem 18.2%, meropenem 17.4%, doripenem 14.4%, and 100 % resistance to cotrimoxazole (Table 2).

Table 2 The antibiotic susceptibility pattern of MDR-PA

Antibiotics	% Sensitivity (n = 110)
Amikacin	80.4
Ciprofloxacin	19.9
Ceftazidime	18.3
Gentamicin	21.8
Imipenem	18.2
Meropenem	17.4
Doripenem	14.4
Piperacilin/Tazobactam	14.00
Cotrimoxazole	0.00

Univariate analysis risk factors for MDR-PA

The univariate analysis indicated that the risk factors for MDR-PA infection were patient-aged more than 60 years old (OR = 2.17, 95%CI = 1.26 - 3.74), patient admitted to the intensive care unit (OR = 2.77, 95%CI = 1.32 - 5.81), duration admitted longer than 7 days (OR_{Adj} = 1.94, 95%CI = 1.13 - 3.32), patient with complications (OR = 3.27, 95%CI = 1.83 - 5.86), patient had an endotracheal tube (OR_{Adj} = 2.44, 95%CI = 1.36 - 4.38), and a mechanical ventilator (OR_{Adj} = 3.04, 95% CI = 1.65 - 5.59) (Table 3).

Table 3 Univariate analysis factors associated with multidrug-resistant *P. aeruginosa* infection

Variable	Cases (n = 110)	Controls (n = 110)	OR_C (95%CI)	*p*-value
	n (%)	n (%)		
Sex				
Male	57 (51.82)	62(56.36)	1.0	
Female	53(48.18)	48(43.64)	1.20(0.70-2.74)	0.499
Age (years)				
≤ 60	49(44.55)	70(63.64)	1.0	
> 60	61(55.45)	40(36.36)	2.17(1.26-3.74)	0.005
Ward admitted to				
Surgical	12(10.91)	14(12.73)	1.0	
Medicine	35(31.82)	32(29.09)	0.83(0.48-1.42)	0.496
Intensive care unit	45(40.91)	50(45.45)	2.77(1.32-5.81)	0.007
Others	18(16.36)	14(12.73)	0.62(0.3 9-1.26)	0.191
Underlying diseases				
No	55(50.00)	63(57.27)	1.0	
Diabetes mellitus	26(23.64)	17(15.45)	0.57(0.28-1.16)	0.122
Hypertension	17(15.45)	13(11.82)	0.67(0.29-1.49)	0.327
Chronic kidney disease	8(7.27)	8(7.27)	0.87(0.30-2.48)	0.799
Cardiovascular disease	2(1.82)	6(5.45)	2.16(0.50-13.51)	0.250
Others	2(1.82)	3(2.73)	1.30(0.21-8.12)	0.772
Complication				
No	25(22.73)	54(49.09)	1.0	
Yes	85(77.27)	56(50.91)	3.27(1.83-5.86)	<0.001
Duration of admitted				
≤ 7 days	50(45.45)	68(61.82)	1.0	
> 7 days	60(54.55)	42(38.18)	1.94(1.13-3.32)	0.015
Surgery				
No	83(75.45)	86(78.18)	1.0	
Yes	27(24.55)	24(21.82)	1.65(0.62-2.18)	0.632
Urinary catheter				
No	71(64.55)	74(67.27)	1.0	
Yes	39(35.45)	36(32.73)	1.12(0.64-1.97)	0.670
Endotracheal tube				
No	64(58.18)	85(77.27)	1.0	
Yes	46(41.82)	25(22.73)	2.44(1.36-4.38)	0.003

Table 3 Univariate analysis factors associated with multidrug-resistant *P. aeruginosa* infection (cont.)

Variable	Cases (n = 110)	Controls (n = 110)	OR_C (95%CI)	*p*-value
	n (%)	n (%)		
Mechanical Ventilator				
No	64(58.18)	89(80.91)	1.0	
Yes	46(41.82)	21(19.09)	3.04(1.65-5.59)	<0.001
Prior antimicrobial receiving				
No	78(70.91)	81(73.64)	1.0	
Yes	32(29.09)	29(26.36)	1.14(0.63-2.06)	0.651

Multivariate analysis of risk factors for MDR-PA

The multivariate analysis indicated the risk factors for MDR-PA infection were: patient aged more than 60 years old (OR_{Adj} = 2.06, 95%CI = 1.14 - 3.71), duration admitted longer than 7 days (OR_{Adj} = 2.43, 95%CI = 1.28 - 4.60), patient had an endotracheal tube (OR_{Adj} = 2.61, 95%CI = 1.42 - 4.80), and a mechanical ventilator (OR_{Adj} = 3.14, 95%CI = 1.67 - 5.88) (Table 4).

Table 4 Multivariate analysis factors associated with multidrug-resistant *P. aeruginosa* infection

Variable	Cases (n = 110)	Controls (n = 110)	OR_C(95%CI)	OR_A(95%CI)	*p*-value
	n (%)	n (%)			
Age (years)					
≤ 60	49(44.55)	70(63.64)	1.0	1.0	0.016
> 60	61(55.45)	40(36.36)	2.17(1.26-3.74)	2.06(1.14-3.71)	
Complication					
No	25(22.73)	54(49.09)	1.0	1.0	0.112
Yes	85(77.27)	56(50.91)	3.27(1.83-5.86)	1.73(0.87-3.43)	
Duration of admittance					
≤ 7 days	50(45.45)	68(61.82)	1.0	1.0	0.006
> 7 days	60(54.55)	42(38.18)	1.94(1.13-3.32)	2.43(1.28-4.60)	
Endotracheal tube					
No	64(58.18)	85(77.27)	1.0	1.0	0.002
Yes	46(41.82)	25(22.73)	2.44(1.36-4.38)	2.61(1.42-4.80)	
Mechanical Ventilator					
No	64(58.18)	89(80.91)	1.0	1.0	<0.001
Yes	46(41.82)	21(19.09)	3.04(1.65-5.59)	3.14(1.67-5.88)	

Note: Adjusted by gender and age; OR_C, Crude odds ratio; OR_A, Adjusted odds ratio; 95% CI, 95% confidence interval; *p*-value from multiple logistic regression

Discussion

The prevalence of MDR-PA infection in Thailand is increasing[8]. However, it is rare to find reports on the risk factors of MDR-PA infection among Thai population. Our study identified that patients aged more than 60 years, who had been admitted to hospital for longer than 7 days and underwent procedures for an endotracheal tube and a mechanical ventilator were important factors for risk of infection. These were consistent with previous studies reporting that hospitalized patients were at high risk of MDR-PA infection[4,9-12]. A study in the United States of America reported that a lower age of patients reduced the risk of MDR-PA infection[11]. This was similar to the finding in the current study. The current study was also consistent with research in Spain that found that the underlying severity of the disease, multi-organ dysfunction syndrome, and severity of acute clinical illness resulted in those patients having a high risk of MDR-PA infection[13,14]. Specifically, the current study found that patients with complications were 1.73 times more at risk of MDR-PA infection. However, patients who had surgery had no risk of MDR-PA infection, which was contrasted by a study in France that found that patients who underwent surgical treatment were 1.9 times more at risk of MDR-PA infection[9]. The current study identified that patients who underwent a procedure with an endotracheal tube and a mechanical ventilator were at high risk of MDR-PA infection, which was consistent with studies reported in Israel, the United States of America, China, and France where a hospital stay and requirements for procedures, mechanical ventilation, and tracheal intubation insertion were important risk factors for MDR-PA infection[4,10,11,15].

Conclusion

Our study findings showed that patients aged more than 60 years and who had been admitted to hospital for more than 7 days and underwent procedures with an endotracheal tube and a mechanical ventilator were important risk factors for MDR-PA infection.

Take home messages

Patients in the high risk should have special care from medical team to prevent MDR-PA infection especially patient that underwent with medical procedures.

Conflicts of interest

The authors declare no conflict of interest.

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Urbanized elderly's requirements for Physical Therapy Clinic

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KEYWORDS
Physical therapy;
Elderly;
Requirement;
Clinic.

ABSTRACT
Although aging society is one of the major problems in Thailand today, this also marks a great opportunity for physical therapy to play a role in the transition of Thai society. The present study aimed to identify the requirements of the elderly who undergo physical therapy treatment at a clinic in the Khon Kaen District, Thailand. Understanding their requirements will be useful in renovating the physical environment and managing the treatment pattern to serve their needs. A total of 127 elderly people were recruited into the study, which was conducted between July 2017 and June 2018. After using a self-administered questionnaire as the study tool, the collected data were analyzed through descriptive statistics. The results revealed that almost all the volunteers were classified as young-old elderly. About 73% of the participants had underlying diseases, of which hypertension was the most common. In addition, the volunteers required a clinic located in a convenient place. Furthermore, they also required facilities with certain features, such as ramps, handrails, and elderly toilets, along with professional therapists and equipment. In conclusion, for the early phase of the aging society, Physical Therapy Clinic should provide suitable physical environment to support the diminishing physical ability of the elderly. Caregiver service while receiving treatment should be provided for the middle-old elderly. Additionally, for the old-old elderly, the transfer service between the residence and the clinic should be offered. Finally, in order to provide the most appropriate assessment and treatment, the geriatric physical therapist should be hired and the sufficient equipment should be provided for the elderly.

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Introduction

In Thailand, the elderly person is someone whose chronological age is 60 years or older[1]. A 2019 report on the Thai elderly estimated that Thailand will become a full-fledged aging society by 2021, as the number of citizens aged 60 or older is expected to increase to 13.1 million, or 20%, of the total population[2]. By the end of 2020, the top five provinces with the highest number of elderly people in Thailand included Bangkok (19.83%), Nakhon Ratchasima (17.98%), Chiang Mai (19.60%), Khon Kaen (18.16%), and Ubon Ratchathani (15.48%), respectively[3]. A study conducted in Thailand reported that at the age of 60, males and females could expect to live, on average, a further 19.1 and 22.6 years, respectively. In addition, the study also indicated that Thai males could expect to spend an average of 18.6 years free from activities of daily living (ADL) disability and the least number of 11.6 years free from instrumental activities of daily living (IADL). For elderly Thai females, who have longer total life expectancy, the average years that they are ADL disability-free and the least number of IADL years are 21.7 and 8.1, respectively[4].

The major problems of the elderly are related to biological aging and the resulting decline in functional capacities. The common diseases among the Thai elderly include musculoskeletal, respiratory, and cardiovascular diseases[5]. Meanwhile, the most prevalent health-related complaints from the elderly have to do with their limited mobility (57.8%), and it has been shown that such complaints tend to increase with age[6]. In order to support age-related health problems, both the government sector (e.g., hospitals or centers of geriatric medicine) and the private sector (e.g., residential homes, elderly hospitals, nursing homes, and home health care services) serve the elderly[5,7,8]. In accordance with this fact together with the nation current situation, there is a tremendous opportunity for physical therapy to play an important role in Thailand's transition into an aging society. In Khon Kaen district, there are 2 public and 3 private hospitals that provide physical therapy services and 6 Physical Therapy Clinics. As known before, Physical therapy council requests the clinic must provide some standard physical environment (e.g. location, usable area, toilet) and treatment equipment (e.g. ultrasonic therapy machine, electrical stimulator, electric traction machine, and hydrocollator unit)[9]. However, the standard requirements for geriatric clinic has not been addressed.

In previous study, the United Nations Population Fund (UNFPA) reported some characteristics between the urban and the rural elderly[10]. It indicated that urban elderly were more educated, more literate and also had more average incomes than the counterparts. They also tended to living in single family style[10]. In addition, as mentioned earlier, Khon Kaen is one of the top five provinces with the highest number of elderly people in Thailand[3]. The year 2021 database report that Khon Kaen province had 291,875 elderly people in which 61,550 elderly lived in the urban area, the Khon Kaen district[11]. Thus, based on this fact, it may influence their requirement when using health services. Therefore, the present study was conducted to identify the requirements of the elderly who undergo physical therapy treatment at a clinic in the Khon Kaen District, Thailand. Understanding these requirements will be useful in planning and renovating the physical environment and managing the treatment pattern and services that can best cater to the needs of the urbanized elderly in the future.

Materials and methods

This study is a descriptive research conducted between July 2017 and June 2018. It was approved by the Khon Kaen University Ethic Committee (HE602176).

Subjects

The elderly aged 60 years and above who visited the Physical Therapy Clinic at the Khon Kaen University Community Outreach Center (KKUCOMOC) for treatment and rehabilitation were invited to join the study. All of them were able to understand verbal communication, read and write Thai, and expressed willingness to participate in the study. At the beginning, 140

elderly people were willing to participate; however, due to much missing information from 13 volunteers, the data analysis was based on a final sample of 127 participants.

Study tool

The study tool was a self-administered questionnaire on the elderly's requirements from an urbanized Physical Therapy Clinic. The questionnaire was based on information from the Ministerial regulations prescribing building facilities for the disabled or handicapped and the elderly[12] and was designed to gather empirical data. It consisted of three sections. The first section consisted of 10 questions that dealt with personal information, including age, gender, marital status, educational level, accommodation characteristics, medical treatment rights, and underlying diseases. The second section was separated into three parts to acquire information about the clinic location and facilities (10 questions), the appointment reminder and transfer assistance service (three questions), and the treatment quality (six questions). The last section enjoined the participants to include their additional suggestions and comments.

For the second section, each elderly person was asked to give opinions evaluating their requirements using a five-point Likert scale ranging from 1 ("least require") to 5 ("strongly require"). In addition, the questionnaire quality was evaluated and the three aspects were focused. Firstly, the content validity was evaluated by content validity index method from three physical therapy professionals. Secondly, the pilot test was conducted in 10 volunteers to assess how well the questionnaire captured the constructs. It was supposed to measure and test the participants' comprehension of the questionnaire items. Lastly, the reliability of each questionnaire item was assessed by calculating the Cronbach's alpha coefficient. In statistical method, the coefficient of Cronbach's alpha and range of reliability interpretation were interpreted as > 0.9 (excellent), 0.85-0.90 (very good), 0.8-0.84 (good), 0.75-0.79 (quite good), 0.70-0.74 (fair) and <0.70 (questionable)[13]. The result of the reliability test in this study was 0.873, which meant that the questionnaire was reliable as an instrument for data collection.

Study procedure

On the day of data collection, the researcher clarified the objectives, benefits, and procedures of the study to the volunteers. Then, the questionnaire was distributed and collected within seven days by asking each participant to drop off their completed questionnaires into the designated return box. Next, the researcher verified the questionnaires in terms of the accuracy and the completeness of the information provided. Then, the answers from 127 out of 140 completed questionnaires were analyzed, representing a response rate of 90%. The results were reported in numbers and percentages of volunteers.

Statistical analysis

The coefficient of Cronbach's alpha was performed with SPSS program version 23 software. In addition, after data collection, the information was verified for the accuracy. Then the data were analyzed and presented as numbers and percentages.

Results

Personal information about the volunteers can be found in Table 1. The data from the first section of the questionnaire indicated that there were more females than males who participated in the study. In terms of the age, the highest number of volunteers were in the 60 to 69-year-old age group. The volunteers showed a diverse range of marital statuses, with married people having the highest proportion among the sample. For the educational level, about 38% of the volunteers had lower than bachelor's degree qualifications. Additionally, government/state enterprise officer was the highest contributing treatment right among the volunteers.

The volunteers' main underlying diseases included hypertension, musculoskeletal disease, cardiovascular disease, and diabetes mellitus. Other underlying diseases were allergies, thyroid disease, gastro-esophageal reflux disease, benign prostatic hyperplasia, and renal disease. Among the volunteers, the major income source was

their pension, and the average medical expenses per month was less than 300 USD for 82% of all volunteers.

Table 2 presents the requirements identified by the elderly while undergoing treatment at the Physical Therapy Clinic. The results revealed that they required a clinic that was conveniently located for easier travel and was designed with a physical environment that was suitable for the elderly. The results from the appointment reminder and transfer assistance service part indicated that the appointment service was the highest requirement among the elderly, followed by caregiver and transfer services. In addition, the result from the treatment quality part indicated that aside from the physical therapist performance, the number and the quality of modern therapy equipment were among the most required by the elderly. These were followed by 1) the staff characteristics; 2) fast, convenient, and equitable service; 3) services that correlate the clinic's announcements; and 4) a channel through which service complaints can be made.

Finally, some interesting insights were obtained from the section on additional suggestions and comments. For example, some volunteers indicated that the clinic should provide non-slip shoes that fit perfectly with their feet. Another volunteer commented that the clinic should provide a security guard who can facilitate parking services and assist the elderly as they get on or off their vehicles.

Table 1 General characteristics of the volunteers (n=127)

Characteristics	Volunteers, n (%)
Gender	
Male : Female	35 : 92 (28% : 72%)
Age (years)	
60–69	87 (68%)
70–79	33 (26%)
80 and over	7 (6%)
Status	
Single	20 (16%)
Married	77 (61%)
Divorce	10 (7%)
Widowed	20 (16%)
Level of education	
Lower than bachelor's	48 (38%)
Bachelor	36 (28%)
Higher than bachelor's	43 (34%)
Living arrangement	
Living alone	16 (13%)
Living with spouse	64 (50%)
Living with son or daughter	32 (25%)
Living with relatives	15 (12%)
Primary caregiver	
No caregiver	14 (11%)
Spouse	47 (37%)
Son or daughter	43 (34%)
Relative	23 (18%)

Table 1 General characteristics of the volunteers (n=127) (cont.)

Characteristics	Volunteers, n (%)
Treatment right	
Self-payment	10 (8%)
Universal health care	7 (5%)
Social security scheme and workmen's compensation fund	2 (2%)
Government/state enterprise officer	108 (85%)
Underlying disease#	
No underlying disease	34 (27%)
Diabetes mellitus	8
Hypertension	38
Cardiovascular disease	12
Musculoskeletal disease	16
Others	39
Income source	
Pension	75 (59%)
Private business	28 (22%)
Other	24 (19%)
Average medical expenses per month	
Less than 300 USD	105 (82%)
300–599 USD	19 (15%)
600–899 USD	2 (2%)
900–1,199 USD	0 (0%)
Over 1,200 USD	1 (1%)

Note: # Volunteers were able to identify more than one underlying disease.

Table 2 Elderly requirements while undergoing physical therapy treatment

Item	Level				
	Strongly required	**Required**	**Neutral**	**Minimally required**	**Least required**
1. Clinic location and facilities					
1.1 Required the convenience travelling location	89 (70)	31 (24)	6 (5)	0 (0)	1 (1)
1.2 Required a ramp with handrails at the main entrance	51 (40)	46 (36)	24 (19)	4 (3)	2 (2)
1.3 The door requirement					
1.3.1 Required extra-wide door leaves	43 (34)	58 (45)	25 (20)	1 (1)	0 (0)
1.3.2 Required sliding and barrier-free doors	60 (47)	51 (40)	13 (10)	1 (1)	2 (2)
1.4 Required a spacious clinic with a sufficient waiting space.	71 (56)	42 (33)	11 (8)	2 (2)	1 (1)
1.5 Required the appropriate lighting illuminance level	58 (45)	53 (42)	15 (12)	1 (1)	0 (0)
1.6 Corridor inside the clinic					
1.6.1 Required flooring with non-slip materials	80 (63)	35 (27)	10 (8)	1 (1)	1 (1)
1.6.2 Required wide and barrier-free corridors	71 (56)	42 (33)	13 (10)	1 (1)	0 (0)
1.6.3 Required wall handrails for walking support	51 (40)	47 (37)	23 (18)	4 (3)	2 (2)
1.7 Required clearly visible and perceptible information guide signs	64 (49)	49 (39)	11 (9)	1 (1)	2 (2)
1.8 Required standard elderly restrooms	75 (59)	38 (30)	13 (10)	1 (1)	0 (0)
1.9 Required standard facilities for the elderly	49 (38)	57 (45)	20 (16)	1 (1)	0 (0)
1.10 Required adequate and convenient parking spaces	103 (81)	22 (17)	2 (2)	0 (0)	0 (0)
2. Appointment reminder and transfer assistance service					
2.1 Required a transfer service between the residence and the clinic	26 (21)	23 (18)	46 (36)	20 (16)	12 (9)
2.2 Required a service allowing for appointment reminders and appointment postponement/ cancellation in advance	40 (31)	49 (39)	34 (27)	3 (2)	1 (1)

Table 2 Elderly requirements while undergoing physical therapy treatment (cont.)

Item	Level				
	Strongly required	**Required**	**Neutral**	**Minimally required**	**Least required**
2.3 Required a caregiver service while receiving treatment if a relative or carer could not be present	32 (25)	46 (36)	(20)	16 (13)	7 (6)
3. Treatment quality					
3.1 Required courteous, polite and eager to serve staff	88 (69)	35 (28)	3 (2)	1 (1)	0 (0)
3.2 Required a qualified and expert physical therapist	102 (80)	22 (17)	2 (2)	1 (1)	0 (0)
3.3 Required modern, adequate, and ready to use physical therapy equipment	101 (79)	23 (18)	2 (2)	1 (1)	0 (0)
3.4 Required fast, convenient, and equitable service	89 (70)	34 (27)	3 (2)	1 (1)	0 (0)
3.5 Required services that correlated the clinic's announcements, such as treatment costs, opening-closing hours, etc.	82 (64)	38 (30)	6 (5)	0 (0)	1 (1)
3.6 Required a channel for service complaints in order to resolve any problem promptly.	56 (44)	50 (40)	18 (14)	3 (2)	0 (0)

Note: Data expressed in number (%).

Discussion

The present study was conducted to identify the requirements of the elderly who undergo physical therapy treatment at a clinic in the Khon Kaen District, Thailand. Identifying and understanding such requirements will be useful in conceiving specific plans and renovating the physical environment and in managing the treatment pattern to better serve the needs of the elderly in the future.

The results of this study indicated some important points. The first point related to the clinic location and facilities. The result indicated that the volunteers strongly required a convenient clinic location for easier travelling, adequate and convenient parking space. Then, the following requirements were flooring with non-slip materials, standard elderly restroom and spacious area both in waiting zone and also the corridor. This finding might be explained by the volunteers' age range. Given that aging affects all physiological processes, the older a person becomes, the greater the effects on his/her functional capacities are. This study found that the majority of the volunteers (68%) who joined the study were in the young-old group (60-69 years), 26% were in the middle-old group (70-79 years), and only 6% (80 years and over) were in the old-old group. Comparatively speaking, the young-old elderly was still in better physical health than the others and was better able to perform self-care activities. Generally, they were able to independently walk up the stairs, go to the toilet, walk on a flat road, walk or drive a car outside the residence, and participate in social activities[14]. Therefore, given their age group, this might be the reason why they rated these requirements.

In addition, based on the results of this study, not only the facilities that mentioned earlier, but also the ramps with handrails at the main entrance, sliding and barrier-free doors, wall handrails for walking support, appropriate lighting illuminance level, and clearly visible and perceptible information guide signs, were strongly required by the elderly. This finding might be related to their underlying disease. The study found that only 27% of the volunteers in this study had no underlying disease. While the remaining 73% had underlying diseases. Among these, hypertension was the most common, followed by musculoskeletal disease, cardiovascular disease, and diabetes mellitus. Specifically, the common musculoskeletal diseases reported were osteoarthritis, osteoporosis, gout, and shoulder, back, and knee pain. According to a previous studies, musculoskeletal diseases often cause diminished movement and ADL functions among the elderly[12,13]. Therefore, the Physical Therapy Clinic should have a building design under the limitations experienced by the elderly.

The second point finding relates to appointment reminder and transfer assistance service. This study found that more than one-third of the participants were neutral in terms of requiring a transfer service between their residences and the clinic, whereas they required an appointment management service and also the caregiver service in case of relative or carer could not be present. As mentioned before, the majority of the volunteers participating in this study were the young-old group that was able to perform self-care activities. Therefore, this finding might be explained why they rated neutral for the transfer service. In addition, this study found that 50% of the volunteers lived with their spouses, 25% lived with son or daughter, and 25% lived alone or with their relatives. The primary caregivers were spouses, and sons or daughters, with almost similar percentages. These results were consistent with the report from a previous study conducted among the elderly from 25 provinces in Thailand[14]. The Suitable Welfare for the Elderly in Different Ages study reported that the majority of the young-old and middle-old elderly lived with their spouses, whereas most of the old-old lived with their daughters[17]. In addition, most of the primary caregivers were daughters living in the same household[17]. In the current study, the elderly volunteers lived in an urban society; half of them lived with their spouses. This might be the reason why the volunteers required or strongly required appointment management service and also required caregiver service if a relative or carer could not be present while receiving the treatment.

Finally, for the treatment quality requirement, this study found that the evaluation items that were strongly required by the elderly were a qualified physical therapist and modern, adequate, and ready-to-use equipment. Based on this finding, the researcher suggests that geriatric physical therapists should be hired to provide more a precise assessment and treatment for the elderly. Additionally, there was another suggestion related to the finding. For the Physical Therapy Clinic that converted from a two-storey commercial building as the clinic at KKUKOMOC, the design features might have certain limitations in serving the requirements of the elderly. Therefore, renovation should be considered. Furthermore, although the underlying diseases found in this study can be classified as non-communicable diseases, they can cause further health problems, such as stroke, chronic ulcer, and falling[14]. Therefore, for a better quality of life, the researchers suggested that the elderly should begin to manage their health before going into old age. Moreover, the government and related agencies should implement relevant policies to prevent the occurrence of these underlying diseases among the elderly population.

Study limitations

Some limitations of this study must be considered. First, the clinic at KKUKOMOC was renovated from a two-storey commercial building; hence, its physical environment might be different from that of other building types. As such, the requirements mentioned in this study might not explain the need for all building types. Second, although requirements of the elderly are likely to be the same for any treatment right; however, 85% of the volunteers in this study cited the government or state enterprise officer treatment right. Based on this finding, they were a largely group of retired government employees with relatively high educational, social, and economical status that may not represent a typical urbanized elderly. Therefore, the results in this study may not fully address the requirements of the elderly using other rights and also the elderly who are typical urbanized. Third, the results of this study explain only the requirements of the elderly who undergo physical therapy treatment at a clinic only. Thus, the requirements of elderly people who have to stay at home or are bedridden cannot be explained by the findings of this study.

Conclusion

For the early phase of an aging society, such as that found in Khon Kaen Province, the Physical Therapy Clinic should provide suitable physical environment that can wholly support the diminishing physical abilities of the elderly. Additionally, for the middle-old elderly, apart from the physical environment, it is important that a caregiver service while receiving treatment should be provided. Furthermore, for the old-old elderly, they should be provided with a transfer service to transport them between the residences and the clinic. Finally, a geriatric physical therapist should be hired, and modern, sufficient equipment should be provided to ensure that the elderly will receive the most appropriate assessment and treatment required.

Take home messages

In order to the transition into an aging society of Thailand, the researchers suggest three points for physical therapy clinic. Firstly, the physical environment should support the diminishing physical abilities of the elderly. Secondly, the physical therapist performance and lastly, the treatment equipment should provide to ensure the treatment efficiency.

Conflicts of interest

The authors declare no conflict of interest.

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The association of medial scapular muscle pain on distance of mouth opening in participants with scapulocostal syndrome: an exploratory study

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KEYWORDS
Myogenic-TMD;
Upper quarter pain syndrome;
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Chronic pain;
Neck pain.

ABSTRACT
Evidence-based studies have reported an association between myogenous-temporomandibular disorder and medial scapular muscle pain. However, the influence of medial scapular muscle pain on distance of mouth opening has still not been investigated. This study aimed to investigate the effect of medial scapular muscle pain on distance of mouth opening in participants with scapulocostal syndrome (SCS). One-hundred and one convenient participants with SCS totaling more than 3 months (14 males and 87 females) were recruited. Participants were divided into three groups consisting of mild, moderate, and severe pain (aged 26.33 ± 1.16, 27.85 ± 0.76, and 27.44 ± 1.10, respectively). The distance of mouth opening was measured in two conditions; pain-free mouth opening and maximum mouth opening. The distance of mouth opening among three groups were compared using Kruskal-Wallis test with Dunn-Bonferroni post hoc analysis. Kruskal Wallis test reported that the distance of maximum mouth opening was significantly different among three groups (p-value < 0.035). Dunn-Bonferroni post hoc found a statistically significant difference in distance of maximum mouth opening between moderate and severe pain groups (p-value = 0.042). In conclusion, medial scapular muscle pain tends to influence maximum mouth opening. Therefore, therapists should assess the distance of maximum mouth opening amid treatment of patients with SCS.

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Introduction

Measurement of distance of mouth opening is a common method used to describe movement pattern and muscle performance in patients with myogenous-temporomandibular disorder (myogenous-TMD) or masticatory myofascial pain (MMP)[1]. Although, most previous studies exhibited association between neck discomfort and increase of severity of symptoms in participants with MMP, none of these studies mentioned the interaction between medial scapular muscle pain and jaw function[2-6].

Various studies found the influence of neck pain on MMP. Kraus (2007), Pasinato et al. (2016), and Padamsee et al. (1994) established that neck pain can increase pain severity in the stomatognathic system[3,6,7]. Zafar et al. (2000) reported that active movement of mouth opening and mouth closing correlated with neck function[8]. Moreover, Hellmann et al. (2012) and Giannakopoulos et al. (2013) reported decreased bite force in MMP patients with neck pain. Previous studies also strongly recommended therapists assess the neck and its structures amid clinical assessment of MMP patients[3,9-11].

The highest prevalence of trigger point (18-30%) was shown in levator scapulae followed by upper trapezius (13-19%)[12]. These muscles provided stability and prevented forward neck flexion, and rotation during static work position, such as using the computer. Likewise, Larsson et al.[13] mentioned that prolonged muscle fiber activation of the levator scapulae and neck extensors during long hours of computer work may lead to development of pain and tenderness. In addition, neck extensor, such as the upper trapezius is innervated by the accessory nerve which attaches to the trigeminal nerve at the trigeminocervical nucleus[14]. Therefore, any injury on the neck muscles may affect the masticatory muscle pain through this connection.

Notably, there are various neck muscles attached to the scapular area. It is well-established that scapular muscles are the main stabilizer of the neck; consequently, some muscle fibers, nerves, and other structures can become concomitantly merged. Any injury to the axioscapular muscle can affect the neck muscles[15,16]. According to this connection, it is difficult for therapists to differentiate the source of pain in patients who report pain at the neck and scapular regions.

Scapulocostal syndrome or SCS concerns pain symptoms exhibited in the scapular area related to abnormal postures in individuals with neck disorders[12,17,18]. Previous studies reported that treatment of the scapular area can improve neck function performance[12,14]. These results implied that the impairment of one part of the body can affect the adjacent areas which lie above or under said structure. Therefore, therapist should also be aware of and assess the adjacent areas of the pathological site. Nevertheless, the influence of medial scapular muscle pain on the distance of mouth opening has not been previously mentioned.

According to the above mentioned, it could be concluded that jaw and neck, and neck and scapula were connected in various aspects. The evidence emphasizes the linkage between the jaw and neck via the sensory-motor-system[19], while the neck and scapula were linked via the axioscapular muscles[15,16]. Moreover, the evidence has also revealed that functional disorders of the upper cervical spine (occiput to C3) are related with the lower cervical spine (C3 to C7) and TMJ[20]. Thus, cervical dysfunction can cause both craniomandibular disorders[6,10,21,22] and the scapular muscles pain. However, the effect of scapular muscles pain on the stomatognathic system is still lacking.

Therefore, this study aimed to investigate the effect of medial scapular muscle pain on distance of mouth opening in patients with SCS. We hypothesized that severity of pain in the medial scapular muscles may associate with the distance of mouth opening in two conditions, i.e. pain-free mouth opening (MO) and maximum mouth opening (MMO) due to masticatory muscle pain.

Materials and methods

Participants

One-hundred and one participants with SCS for more than 3 months were recruited. Patients' subjective information regarding history of medial

scapular muscle pain, pain duration, and working duration were collected. Inclusion criteria were male or female aged between 18 to 50 years who reported chronic pain symptom in the medial scapular muscles along with the medial scapular area at not less than 3 points with a specific referral pattern followed by the description of trigger point by Travell and Simon (1999)[23]. Participants were excluded if they reported a history of TMD with disc displacement, TMJ osteoarthritis, a history of serious systemic disease or serious condition such as fracture, trauma, surgery of the cervical spine, or inflammatory disease.

Participants were allocated into 3 groups based on severity of pain intensity during receiving pressure in the medial scapular muscles, based on a mild (VAS = 0-30 mm.), moderate (VAS = 40-60 mm.), and severe pain scale (VAS = 70-100 mm.). Participants were recruited via advertisement and those who met inclusion criteria were informed of the purposes and procedures. Consequently, they were asked to sign a consent form. The study was conducted at Research Room 5 in the Faculty of Associated Medical Science, Khon Kaen University, Thailand. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE 612318).

Measurements

This research used a visual analog scale (VAS) to assess pain intensity in the most painful areas of the medial scapular muscles consisting of the levator scapulae, rhomboid minor and major, serratus posterior superior, and upper trapezius muscles. The assessment position required subjects to be in the sitting position with shoulder adduction and internal rotation with the assessor placing their hand on the opposite shoulder to the affected side (Figure 1). The assessor applied compression force (2 kg/cm^2) along the medial scapular area which is the insertion point of the medial scapular muscles. Participants were measured for the tender spot along the medial scapular muscles for at least three points in order to represent pain of those five muscles mentioned above. During the assessment period, participants were asked to explain the pattern of muscle referred pain and asked to select the most painful area. The selected point was used to assess the pain intensity. The reliability and construct validity of VAS were reported to be high (r = 0.99 and gamma = 0.77 to 0.89, respectively)[24,25]. Whereas, the reliability of the palpation in the diagnosis of myofascial pain syndrome and myofascial trigger point was reported to be good (K = 0.81), and the validity showed high values of sensitivity and specificity (sensitivity = 0.92, specificity = 1.00)[26].

Figure 1 Assessment position for pain intensity in the medial scapular muscles

The TheraBite™ was used to measure the distance of mouth opening for both conditions. For the pain-free mouth opening (MO) condition, participants were asked to open their mouth without pain or discomfort in masticatory muscles. Whereas, for the maximum mouth opening (MMO) condition, participants were asked to open their mouths as wide as possible even if they felt pain in the masticatory muscles. The distance of mouth opening was measured by the distance between the incisal edges of the upper and lower central incisors. This method was reported to present excellent reliability amid both measurements by a therapist (r = 0.90 to 0.96)[27] and by patient self-measurement (r = 0.92)[28].

Painful areas were marked on a clear-plastic sheet by the researcher to guide the testing area for the assessor in order to perform pain intensity testing. Outcome measurements were assessed amid 3 repetitions. Participants were asked to rest for 1 to 2 minutes between each test.

Statistical analysis

Descriptive statistics were performed on demographic data and presented as means, standard deviations (SDs) and medians. Kolmogorov-Smirnov showed non-normal distribution of pain intensity in the medial scapular muscles and mouth opening distance for both conditions; therefore, the non-parametric (Kruskal–Wallis) test was conducted to determine the differences in distance of mouth opening conditions among groups. Dunn-Bonferroni test was used for pairwise comparison. The author employed SPSS version 23.0 for Windows for data collection and data analysis. Significance level was set at *p*-value < 0.05.

Results

Demographic data, health status, and baseline characteristics

One hundred and one convenient participants with SCS for greater than 3 months (14 males, 87 females) were recruited and screened by the researcher from July 2019 to September 2020. Subjects were divided into three groups consisting of mild, moderate, and severe pain of the medial scapular muscles. Eighteen had mild pain, 58 had moderate pain, and 25 had severe pain (mean age (SD): 27.44 ± 1.10 years). Baseline characteristic data of the three groups are summarized in Table 1. There were no significant differences in baseline characteristic data among three groups. The results demonstrated that levator scapulae muscle was the most painful spot (77.2%) followed by rhomboids, and serratus posterior superior, respectively (Table 2).

Table 1 Baseline characteristics of the three groups: mean (SD)

Characteristic	Mild pain (n=18)	Moderate pain (n=58)	Severe pain (n=25)	*p*-value
Age (years)	26.33 ± 1.16	27.85 ± 0.76	27.44 ± 1.10	0.520
Weight (kg)	59.56 ± 3.28	58.10 ± 1.64	58.76 ± 2.43	0.846
Height (cm)	163.94 ± 1.66	161.19 ± 0.80	161.24 ± 1.69	0.080
BMI (kg/m^2)	22.07 ± 1.01	22.31 ± 0.58	22.53 ± 0.79	0.982
Working age (months)	35.89 ± 13.87	37.40 ± 7.66	37.12 ± 8.53	0.725
Working duration (hours)	5.28 ± 0.99	6.28 ± 0.50	6.40 ± 0.98	1.381
Weekly day (days)	3.78 ± 0.69	4.29 ± 0.32	4.24 ± 0.56	0.198
Pain duration (months)	18.83 ± 5.04	31.47 ± 4.49	26.48 ± 5.79	0.091
Pain of SCS	2.97 ± 0.17	5.28 ± 0.12	8.06 ± 0.13	0.038*

Note: Homogeneity test of variance, *p-value < 0.05

Table 2 Frequency of medial scapular muscle pain in all participants

Medial scapular muscle	N = 101	Percent (%)
Levator scapulae	78	77.2
Rhomboids	20	19.8
Serratus posterior superior	3	3
Upper trapezius	0	0

Distance of mouth opening

Median scores of distance of mouth opening are shown in Table 3. Kruskal-Wallis test reported that there was a statistically significant difference in distance of maximum mouth opening in terms of pain severity variations in the medial scapular muscle (p-value < 0.035) (see Figure 2). Dunn-Bonferroni test showed statistically significant differences between the moderate and severe pain groups. Distance of maximum mouth opening was significantly lower in SCS patients with severe pain (median of VAS score = 37.86) than those with moderate pain in the medial scapular muscles (median of VAS score= 55.09, p-value = 0.042) while the other groups' comparison did not showed any different significance (severe pain vs mild pain, p-value = 0.132, moderate pain vs mild pain, p-value = 1.000).

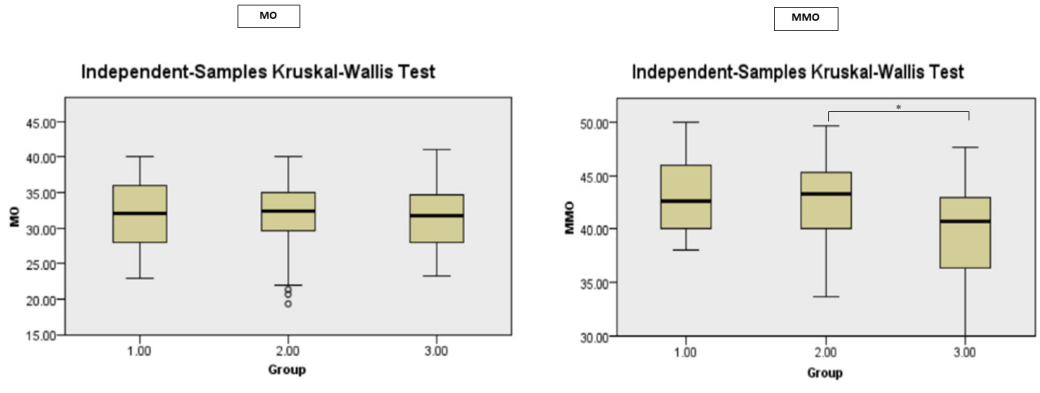

Figure 2 Comparisons of distance of mouth opening in terms of differences in pain severity variations among participants with SCS were presented as median score (mm)

Table 3 Distribution and median scores for distance of mouth opening (both MO and MMO) regarding severity of pain in the medial scapular muscles

Group/mouth distance	MO Mean rank (mm)	MMO Mean rank (mm)
Mild pain (n = 18)	53.06	56.08
Moderate pain (n = 58)	51.93	55.09
Severe pain (n = 25)	47.36	37.86

Discussion

To our knowledge, this is the first study to examine the effect of severity of pain intensity in the medial scapular muscles on the distance of mouth opening. The authors hypothesized that the distance of pain-free mouth opening and the distance of maximum mouth opening conditions were different among groups. The results of this study support the hypothesis that distance of mouth opening is affected by pain severity in the medial scapular muscles.

Even though the upper trapezius muscle lies covering the other medial scapular muscles, somatic referred pain pattern can be present at the medial scapular site. The most painful area experienced by SCS participants is presented in the levator scapulae muscle. However, the influence of pain in the medial scapular muscles was significantly presented in the maximum mouth opening condition only. The authors suggest that in pain-free mouth opening, only the masticatory muscles themselves suffice to maintain their function and neutrality. Whereas, in the maximum mouth opening condition, the demand from other muscles which lie in adjacent areas are needed.

The mechanics of mouth opening consists of three mandible motions, including depression, protrusion, and lateral excursion(29). Mandibular depression occurs with combination of rotation (approximately 25 mm) and anterior translation (approximately 15 mm) in the pain-free mouth opening (40 mm). Protrusion occurs with translation and minimal rotation of the mandibular condyle, whereas lateral excursion occurs with ipsilateral rotation and contralateral translation of the mandibular condyles[29]. In 2018, Wang et al. reported the effect of sustained maximum mouth opening in a novel mouse model of TMD[30]. The results demonstrated that sustained maximum mouth opening is risk factor of chronic TMD and inflammation associated with macrophage and microglia in the tissue (such as masseter muscle) and trigeminal system.

Furthermore, there is supportive information confirming the functional linkage between the human stomatognathic system and craniocervical region. In more detail, functions of the masticatory and neck muscles are automatically synchronized via neutral command of the motor systems during opening and closing of the mouth[8]. Moreover, a previous study mentioned that the mandible and neck concomitantly work amid the maximum mouth opening condition[8]. Notably, the neck muscles attached to the scapular region may affect jaw function as well.

In this study, the levator scapulae was the main structure to be focused because it was the most frequently reported painful in the results. A previous study reported that tenderness of the levator scapulae and upper trapezius were major sources of neck pain (18-30% and 13-19%, respectively)[12]. According to the myofascial linkage concept, muscles and fascia in the human body are connected in series, consequently, each muscle can influence more than one joint[31]. As a result, pain intensity amid these muscles may transfer to the neck and disturb jaw function, especially, maximum mouth opening. In this study, the results revealed that distance of maximum mouth opening was significantly lower in SCS patients with severe pain than those with moderate pain in the medial scapular muscles.

This study also discovered a significant impact of medial scapular muscle pain on distance of mouth opening. Although this study revealed impact of pain intensity on distance of mouth opening, reports of the positive impacts rendered by treatment in a clinical setting are still lacking. Henceforth, the authors suggest that the development of a treatment program which simultaneously focuses on SCS and jaw function would be worthwhile.

Unfortunately, there were some study limitations. Since, the nature of MMP is that of a fluctuating disorder, the findings may have been affected by activities of daily living, the time of assessment, and pain intensity in the masticatory muscles during the study period. Moreover, this study did not assess other jaw functions, such as bite force, yawning, and so on. Notably, future studies focusing on the association between SCS and other functions of the mandibular system are needed.

Conclusion

In conclusion, medial scapular muscle pain tends to influence distance of mouth opening, especially with regards to the maximum mouth opening condition. Notwithstanding, the masticatory muscles can maintain mandible neutrality during chewing or mastication. Pain and distance of maximum mouth opening tend to be affected by medial scapular muscle pain. Therefore, therapists ought to assess the distance of maximum mouth opening in the treatment of patients with SCS.

Take home messages

Individuals presenting chronic neck or scapular pain may experience a limited distance of mouth opening.

Conflicts of interest

The authors declare no conflict of interest.

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