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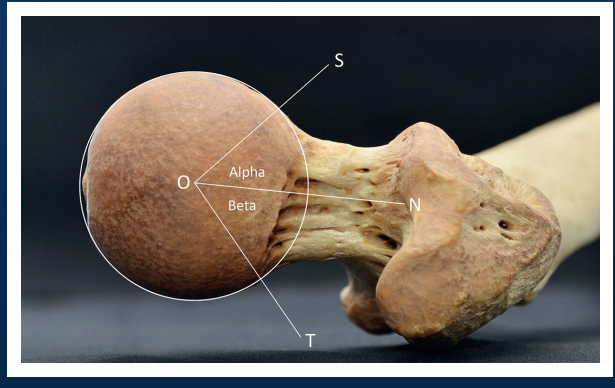
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Cefoxitin Plus Doxycycline Versus Clindamycin Plus Gentamicin in Hospitalized Pelvic Inflammatory Disease Patients: An Experience from A Tertiary Hospital

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ABSTRACT

Objective: To compare length of hospital stay (LOS) and surgical rate in patients hospitalized with pelvic inflammatory disease (PID) who received either cefoxitin plus doxycycline regimen or clindamycin plus gentamicin regimen.
Methods: Medical records of patients hospitalized with PID from 2004 to 2011 were reviewed. Study population was women aged 14-40 years old who had a first-time, admitted diagnosis and a discharged diagnosis of PID. Patients who had prior hysterectomy, bilateral salpingectomy and were not sexually active were excluded. The patients received either intravenous cefoxitin (2 grams every 6 hours) plus oral doxycycline (100 mg twice a day) regimen or intravenous clindamycin (900 mg every 8 hours) plus gentamicin (240 mg once daily) regimen. Outcomes of interest were LOS and surgical rate.
Results: Of 252 eligible participants, 141 (55.95%) received cefoxitin plus doxycycline and 111 (44.05%) received clindamycin plus gentamicin. The patients receiving cefoxitin plus doxyclycline had statistically significant lower age and less number of cases of tubo-ovarian abscess (TOA) ($P<0.05$). Logistic regression showed the similar LOS and surgical rate in both groups after adjusted with age and TOA. No severe adverse effect was identified in both regimens.
Conclusion: Cefoxitin plus doxycycline regimen appears as effective as clindamycin plus gentamicin regimen for treating hospitalized PID patients in terms of LOS, surgical rate and safety profile.

Keywords: Antibiotics; cefoxitin plus doxycycline; clindamycin plus gentamicin; pelvic inflammatory disease; tubo-ovarian abscess (Siriraj Med J 2018;70: 479-483)

INTRODUCTION

Pelvic inflammatory disease (PID) is a common disease in reproductive-aged women.[1,2] Its prevalence was about 2.2 % of reproductive US population.[1] At Siriraj Hospital, the biggest tertiary hospital in Thailand, PID accounts for 3% of admitted gynaeclogical patients per year.[3] PID is a combination of two of these following clinical manifestations: endometritis, salpingitis, oophoritis, tubo-ovarian abscess and pelvic peritonitis.[4-5] PID was diagnosed by one or more of the minimum criteria: cervical motion tenderness, uterine tenderness or adnexal tenderness plus one or more of the additional criteria: oral temperature

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>38.3°C, cervical mucopurulent discharge, presence of numerous WBC on microscopy of vaginal fluid, elevated erythrocyte sedimentation rate (ESR), elevated C-reactive protein (CRP) and laboratory documentation of cervical infection with N. gonorrhoeae or C. trachomatis.[4] The most common cause of PID is sexually transmitted bacterial infection, especially *Neisseria gonorrhea and Chlamydia trachomatis*.[6,7] These are also the most common pathogens of PID in Thai women.[8] However, a variety of other causative agents have been reported.[9-14] Owing to the frequent co-incidence of anaerobic bacteria, broad-spectrum antibiotics are required.[4] Late commencement as well as incomplete course of antibiotics has shown some association with infertility, chronic pelvic pain and ectopic pregnancy.[15-18]

Intravenous (IV) antibiotic regimen is recommended for patients with tubo-ovarian abscess (TOA), severe clinical signs, PID during pregnancy and poor response to out-patient regimen.[4] Due to higher severity of these conditions, their antibiotic regimens differ from those treated out-patiently.[4] Following the recommendations of the Center for Disease Control and Prevention (CDC)[4], the current first-line regimens are cefoxitin plus doxycycline or clindamycin plus gentamicin. Broad spectrum cephalosporins such as cefoxitin and ceftriaxone effectively cover *Neisseria gonorrhea*, but they fail to treat *Chlamydia trachomatis* and gram-negative anaerobes. Therefore, doxycycline must be added. Clindamycin has a high efficacy for treating anerobic bacteria whereas gentamycin is primarily used against gram-negative bacteria. As a result, clindamycin plus gentamicin regimen is more preferable for treatment of patients with TOA. However, a meta-analysis based on in-patient PID cases showed a similar cure rate between cefoxitin/cefotetan plus doxycycline and clindamycin plus gentamicin, at 92-94%.[19] At the moment, neither of the regimens has shown clear superiority.[20] Thus, the present study aims to demonstrate our experience in using these two regimens in Siriraj Hospital.

MATERIALS AND METHODS

Study design and setting

The cross-sectional study was conducted by reviewing medical charts of consecutive patients being admitted with PID in Siriraj Hospital from 2004 to 2011. Ethical approval was issued by the Siriraj Institutional Review Board (Si 272/2014).

Participants

This study is a part of our published work "Reproductive outcomes of patients being hospitalized with pelvic inflammatory disease"[18] which focused on the long-term sequelae of PID. Inclusion criteria were being 14-40 year-old women with first-time diagnosis with PID and a similar discharge diagnosis (PID or TOA). All patients underwent transvaginal ultrasonography at outpatient department. Indications of hospitalization were in accordance with the CDC guideline, including tubo-ovarian abscess (TOA), severe clinical signs, PID during pregnancy and poor response to out-patient regimen.[21] Women who had undergone hysterectomy, bilateral salpingectomy with or without oophorectomy, and who were not being sexually active were excluded.

Antibiotics regimen

Following CDC 2002 guideline[21], the recommended antibiotic regimens of which were similar to those of the current CDC guideline, either cefoxitin (2 grams every 6 hours) plus doxycycline (100 mg twice daily) regimen or clindamycin (900 mg every 8 hours) plus gentamicin (240 mg once daily) regimen were prescribed for in-patient PID cases in Siriraj Hospital. Re-evaluation of clinical response was routinely done at 48-72 hours after initiating the antibiotics.

Outcome measurement

Outcomes of interest were length of hospital stay (LOS) and need for surgical intervention. LOS >6 days is a poor prognostic indicator of PID.[22,23] Surgical treatment usually resulted from severe conditions including unstable vital signs and worsening of clinical signs.[24]

Statistical analysis

Stata version 12.1 (StataCorp LP, TX) was used to describe participants' characteristics and to demonstrate the comparison between two regimens. Descriptive statistics such as frequency (percentage), mean ± S.D. and median (min, max) were used. Chi- square test and T-test were used for the comparison. Logistic regression was used to explore association between antibiotic regimen and outcomes of interest. P value <0.05 was considered statistically significant.

RESULTS

Of 252 eligible patients, 141 (55.95%) received cefoxitin plus doxycycline and 111 (44.05%) received clindamycin plus gentamicin. Those who received cefoxitin plus doxycycline had the mean age of 24.5±9.3 years, which was significantly less than clindamycin plus gentamicin group (28.0±10.7 years) (p <0.05). Additionally, cefoxtin plus doxycycline group had significantly lower incidence of TOA (12 (8.5%) vs. 64(57.7%), p <0.001).

Other characteristics were comparable between groups, including body mass index (BMI), parity, abortion, high fever and detection of *N. gonorrhea*. Almost all participants had pelvic pain as the presenting symptom. (Table 1)

Patients in cefoxitin plus doxycycline group had lower surgical rate (4.3% vs. 8.1%) and LOS >6 days (6.4% vs. 21.6%) (Table 2). However, after adjusting for age and TOA, there was no significant association between antibiotic regimen and the outcomes of interest (p >0.05). Surgical interventions included three cases of diagnostic laparoscopy and three cases of unilateral salpingo-oophorectomy in cefoxitin plus doxycycline group, and four cases of total abdominal hysterectomy, two cases of unilateral salpingo-oophorectomy and three cases of diagnostic laparoscopy in clindamycin plus gentamicin group. No severe adverse effect of either regimen was noted.

DISCUSSION

The present study re-visits the efficacy of the two intravenous antibiotic regimens recommended by the CDC. Most baseline characteristics were comparable except that cefoxitin plus doxycycline group tended to be initiated in patients with younger age and lower severity of PID. Despite that, when taking age and TOA into consideration, cefoxitin plus doxycycline regimen and clindamycin plus gentamicin regimen resulted in similar outcomes of interest which were surgical rate and LOS >6 days. The findings were compatible with previous studies.[25-28] Moreover, like a recent Cochrane systematic review of RCTs comparing clindamycin plus aminoglycoside versus cephalosporin, the present study demonstrated no difference in adverse effects.[20]

In addition to the findings of the present study, our previous work on the same cohort reported no re-admission owing to persistent PID.[18] This contrasts to

TABLE 1. Baseline characteristics.

	Cefoxitin+Doxycycline (N=141)	Gentamicin+Clindamycin (N=111)	P-value
Age (years)	24.5±9.3	28.0±10.7	0.006
BMI (kg/m^2)	20.6±3.6	21.7±4.2	0.498
Parous women	61 (43.3%)	53 (47.8%)	0.478
Previous abortion	41 (29.1%)	35 (31.5%)	0.674
Body temperature >38.3°C	44 (31.2%)	43 (38.7%)	0.289
Pelvic pain as a chief complaint	135 (95.7%)	107 (96.4%)	0.792
Tubo-ovarian abscess	12 (8.5%)	64 (57.7%)	<0.001
Detection of *N. gonorrhea*	14/48 (29.2%)	11/48 (22.9%)	0.485

TABLE 2. Logistic regression of antibiotic regimen and outcomes.

	Cefoxitin+ Doxycycline (N=141)	Gentamicin+ Clindamycin (N=111)	P-value	cOR (95%CI)	aOR* (95%CI)
Surgical rate	6 (4.3%)	9 (8.1%)	0.199	0.50 (0.17-1.46)	0.87 (0.24-3.19)
LOS >6 days	9 (6.4%)	24 (21.6%)	<0.001	0.25 (0.11-0.56)**	0.44(0.17-1.15)

*Adjusted for age and tubo-ovarian abscess, **p=0.001

Abbreviations: aOR = adjusted odd ratios, cOR crude odd ratios, LOS = length of hospital stay

a previous study which showed a clinical cure rate at around 70 % in TOA cases treated with clindamycin plus gentamicin.[29] Cefoxitin, a third generation cephalosporin is a first-line drug for treating gonorrhea while doxycycline is used to treat chlamydial infection. For uncomplicated PID, this regimen has >90% efficacy.[25-28] Patients with TOA, which is a predictive factor of medical treatment failure[23], tended to receive clindamycin plus gentamicin due to the property of clindamycin against anaerobic bacteria and that of gentamicin for treating gram-negative bacteria. Some evidence showed that gentamicin acts synergistically with clindamycin in the treatment of *C. trachomatis*.[30] The present study supports that cefoxitin plus doxycycline can also be used effectively in patients with TOA.

Parenteral antibiotics for PID which are recommended by the CDC included Cefotetan or cefoxitin plus doxycycline, clindamycin plus gentamicin and ampicillin/sulbactam plus doxycycline.[4] Cephalosporins have been used for a long time and shown supporting evidence in treating PID, although when anaerobic bacteria infection is suspected, cefoxitin appeared more favorable.[31] Ampicillin/sulbactam plus doxycycline is an alternative regimen because of its impressive outcomes in PID patients with inflammatory masses[32] and acceptable safety.[33] Another guideline by the International Union of Sexually Transmitted Infections (IUSTI) has the same recommended first line intravenous antibiotic regimen, but different alternative regimen.[6] Instead of ampicillin/sulbactam, ofloxacin or ciprofloxacin plus metronidazole is classified as the alternative regimen.[6] Previous randomized clinical trials regarding fluoroquinolones and PID, have shown 100% efficacy in eradicating *N. gonorrhea and C. trachomatis*, but lack of anaerobic bacteria coverage. However, *N. gonorrhea* resistant to fluoroquinolone is ubiquitous in Southeast Asia.[34] World Health Organization (WHO) Gonococcal Antimicrobial Surveillance Programme (GASP) has shown that the percentage of gonococcal resistance to ciprofloxacin was 70-100% in Thailand[34], so it is not included in the local guideline.

The strengths of the present study are the homogeneity of participants in terms of ethnicity, severity, their first-time diagnosis of PID and similar discharge diagnosis. Selection bias might be diluted from the fact that all consecutive cases of patients being hospitalized were included. Nonetheless, the primary outcome was not considered from the beginning of the study. Therefore, more severe cases were in clindamycin plus gentamicin group. The well-designed comparative studies between these two regimens are needed as well as novel regimens should be further explored.

In conclusion, clindamycin plus gentamicin regimen is as effective as cefoxitin plus doxycycline regimen in terms of the length of hospital stay and surgical rate among patients hospitalized with PID.

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Digital Photographic Measurements of the Proximal Femoral Anatomy

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ABSTRACT

Objective: This study evaluated the morphology of the proximal femur in the Thai population, especially the femoral head-neck relationship and the femoral neck-shaft relationship, using digital photographs. **Methods:** The morphology of each proximal femur was evaluated via the standard anteroposterior and cephalocaudal views of digital photographs. The femoral head-neck junction morphology was evaluated for translation, rotation, and concavity. The femoral neck-shaft anatomy was measured with the neck-shaft angle and the neck version. **Results:** Fifty-nine Thai femora with an average age of 45.4 years were evaluated. For the femoral head-neck translation, the average superior/inferior offset ratio was 1.01, and the average anterior/posterior offset ratio was 0.97. For the femoral head-neck rotation, the average anteroposterior and lateral physeal angles were 77.5° and 81.9°, respectively. Regarding the head-neck junction concavity, the average gamma, delta, alpha, and beta angles were 45.6°, 44.5°, 45.5°, and 36.2°, respectively. The average femoral neck-shaft angle was 129.5°, and the average femoral neck version was 5.7° of anteversion. **Conclusion:** Thai femora had their heads located almost at the center of the femoral neck, and they were rotated in abduction and anteversion relative to the neck axis. The femoral head-neck junctions showed less concavity anteriorly and superiorly.

Keywords: Digital photograph; femoral head-neck junction; femoral neck-shaft angle; femoroacetabular impingement syndrome; proximal femoral anatomy (Siriraj Med J 2018;70: 484-489)

INTRODUCTION

The morphology of the proximal femur has been evaluated to explain the biomechanics of the normal hip and pathophysiology of various hip disorders.[1-3] Some conditions with an abnormal morphology of the proximal femur (especially the relationships between the femoral head, neck, and shaft) can be the causes of early osteoarthritis of the hip.[1] Femoroacetabular impingement (FAI) syndrome is a hip disorder caused by abnormal morphologic features of the proximal femur (cam morphology) and/or the acetabulum (pincer morphology).[4] Repetitive abnormal contact between the femoral head-neck junction and the acetabular rim results in chondral and labral damage.[5] These chondral and labral lesions cause groin pain and contribute to the development of early osteoarthritis of the hip.[1,5]

The cam morphology of the proximal femur is characterized by the presence of a flattening or convexity at the femoral head-neck junction.[4,5] This abnormality can be identified with plain radiographs, CT scans, and MRIs.[6-8] A prospective cohort by Agricola et al.[9] demonstrated that patients with a higher severity of cam

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morphology had higher adjusted odds ratios in progression to end-stage osteoarthritis. Regarding FAI syndrome, the femoral neck-shaft relationship also determined the degree of labral damage. Ejnisman et al.,[10] revealed that patients undergoing hip arthroscopy for FAI syndrome who had greater femoral anteversion were more likely to have larger labral tears and more anterior extension of the labral lesions.

Previous studies of the morphology of the proximal femur of Thais usually focused on the femoral neck and proximal shaft dimensions for orthopedic implant designs[11] and hip fracture risk assessments.[12,13] Digital photographs have been used to evaluate the proximal femoral anatomy of Caucasian and African-American skeletons.[3,14] The purpose of this study was to evaluate the morphology of the proximal femur in Thai skeletons, especially the femoral head-neck relationship and the femoral neck-shaft relationship, using digital photographs.

MATERIALS AND METHODS

Samples of Thai femora were obtained from the Department of Anatomy, Faculty of Medicine Siriraj Hospital. Exclusion criteria were aged less than 20 years at the time of death, and femora with an obvious abnormal appearance by gross visual inspection (such as osteoarthritis, osteonecrosis, previous fractures, and other deformities). One femur was randomly selected from each human skeleton.

Each specimen was photographed with a digital camera (Nikon D7000; Nikon, Tokyo, Japan) in 2 standard positions: the anteroposterior (AP) and the cephalocaudal views. For the AP photographs, each femur was placed in a supine position on a flat table, with the anterior surface pointing to the ceiling. The femoral neck was then positioned parallel to the table surface. If the neck axis was anteverted, the femoral shaft was rotated internally, using paper cards to increase the support under the lateral femoral condyle, until the neck axis was parallel to the table surface by cephalocaudal inspection. The femur was rotated externally by supporting the medial condyle if the neck axis was retroverted. The AP photographs were then taken from the direct overhead position, perpendicular to the femoral neck axis.

For the cephalocaudal photographs, each femur was placed on a flat table with the anterior surface facing up, and both femoral condyles resting on the table surface. The femoral shaft was abducted until the neck axis was parallel to the edge of the table by overhead inspection. The cephalocaudal photographs were taken with the camera's view that was parallel to the floor and perpendicular to the femoral neck.

The AP and cephalocaudal photographs of all specimens were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The femoral head-neck relationship was evaluated by measurements of translation, rotation, and concavity.[3] The femoral neck-shaft relationship was assessed by the neck-shaft angle and neck version measurements.[3,14]

Femoral head-neck translation refers to the amount of femoral head translation on the femoral neck in the direction that is perpendicular to the neck axis.[3,15] The superior (SOS) and inferior (IOS) offsets were measured in the AP pictures (Fig 1), while the anterior (AOS) and posterior (POS) offsets were obtained from the cephalocaudal pictures (Fig 2). The amount of offset in each direction was the minimum distance between a line drawn parallel to the femoral neck axis and tangential to the femoral head, and a parallel line tangential to the most concave point of the femoral neck. These 4 offset measurements were used to calculate 2 offset ratios: AOS/POS ratio, and SOS/POS ratio. In addition, the anterior head-neck offset ratio was the proportion between the AOS distance and the femoral head diameter measured in the cephalocaudal view (Fig 2).[16]

The femoral head-neck rotation was characterized by the orientation of the physeal scar at the head-neck junction with respect to the femoral neck axis.[3] In the AP photographs, the AP physeal angle was the supero-lateral angle between the line of the anterior physeal scar and a line representing the neck axis (Fig 3). In the

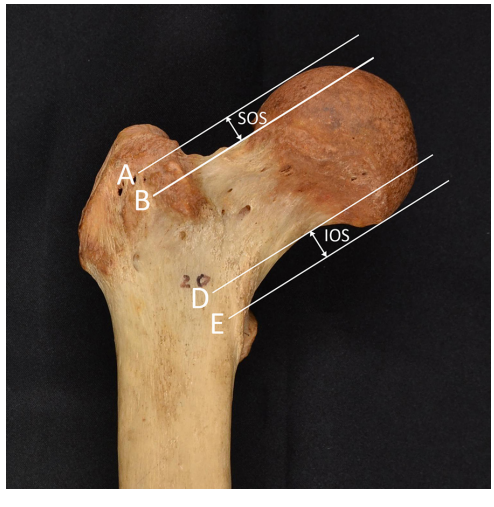


Fig 1. For the femoral head-neck translation, measured in the AP photographs, the superior offset (SOS) was the minimum distance between a line drawn parallel to the femoral neck axis and tangential to the femoral head (line A), and a parallel line tangential to the most concave point of the superior aspect of the femoral neck (line B). The inferior offset (IOS) was the minimum distance between lines D and E, which were located inferiorly. If the SOS/IOS ratio was less than 1, the femoral head translated inferiorly from the femoral neck axis.

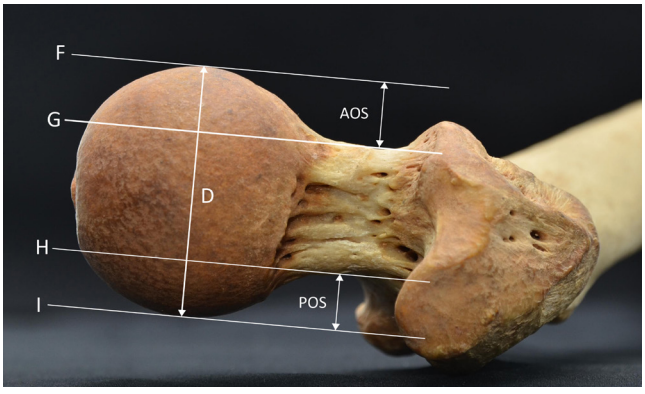


Fig 2. For the femoral head-neck translation, measured in the cephalocaudal photographs, the anterior offset (AOS) was the minimum distance between a line drawn parallel to the femoral neck axis and tangential to the femoral head (line F), and a parallel line tangential to the most concave point of the anterior aspect of the femoral neck (line G). The posterior offset (POS) was the minimum distance between lines H and I, which were located posteriorly. If the AOS/POS ratio was less than 1, the femoral head translated posteriorly from the femoral neck axis. In addition, the anterior head-neck offset ratio was the proportion between the AOS distance and the femoral head diameter (D).

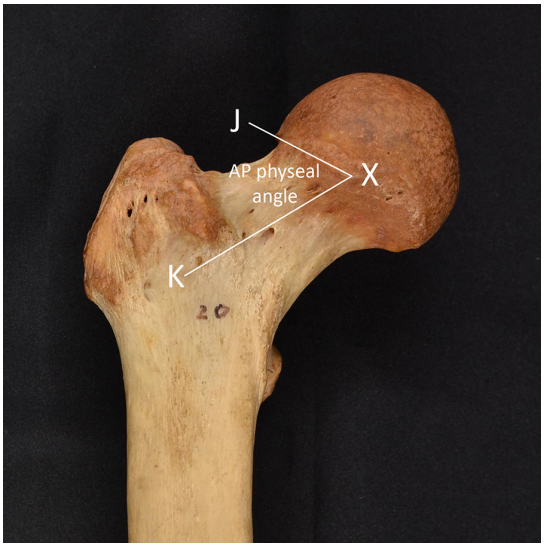


Fig 3. The AP physeal angle, measured in the AP photographs, was the supero-lateral angle between the line of the anterior physeal scar (line XJ) and a line representing the neck axis (line XK). If the AP physeal angle was less than 90 degrees, the femoral head was abducted with respect to the femoral neck axis.

cephalocaudal photographs, the lateral physeal angle was the anterosuperior angle between the line of the superior physeal scar and a line representing the neck axis (Fig 4).

The femoral head-neck junction concavity was created from a near spherical femoral head joining a femoral neck. This parameter was quantified in each direction with a measurement that was modified from the alpha

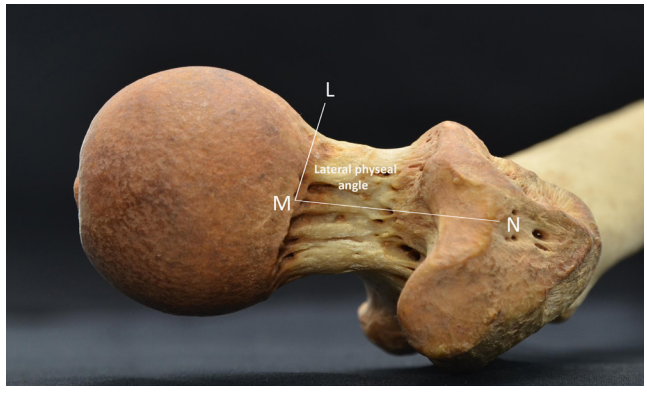


Fig 4. The lateral physeal angle, measured in the cephalocaudal photographs, was the antero-lateral angle between the line of the superior physeal scar (line LM) and a line representing the neck axis (line MN). If the lateral physeal angle was less than 90 degrees, the femoral head was anteverted with respect to the femoral neck axis.

angle measurement method for MRIs described by Nötzli et al.[7] The alpha angle is the angle between the femoral neck axis and a line from the femoral head center to the point marking the beginning of the asphericity of the anterior head-neck junction. Using the same measurement method, the femoral head-neck junction concavity was measured via the gamma and delta angles, superiorly and inferiorly, in the AP photographs (Fig 5). In addition, the alpha and beta angles were measured anteriorly and posteriorly in the cephalocaudal photographs (Fig 6). Smaller angles for these parameters represented more concavity of the femoral head-neck junctions.

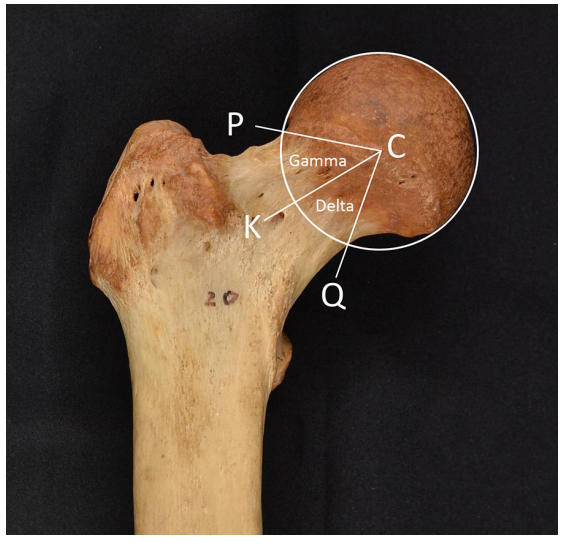


Fig 5. For the femoral head-neck concavity, measured in the AP photographs, the gamma angle was the angle between the femoral neck axis (line CK) and a line from the femoral head center (C) to the point marking the beginning of the asphericity of the superior head-neck junction (line CP). The delta angle was the angle between lines CK and CQ, which represented the concavity of the inferior head-neck junction.

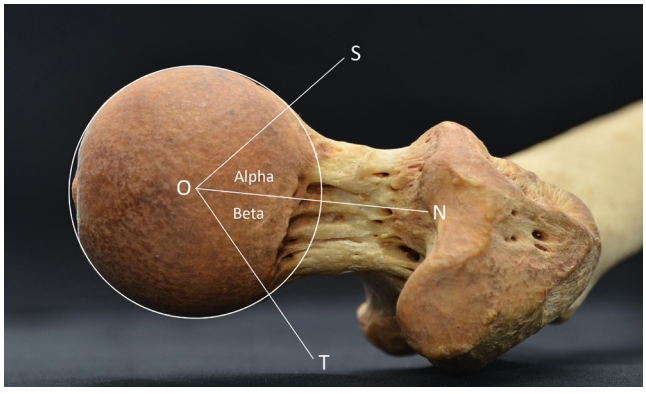


Fig 6. For the femoral head-neck concavity, measured in the cephalocaudal photographs, the alpha angle was the angle between the femoral neck axis (line ON) and a line from the femoral head center (O) to the point marking the beginning of the asphericity of the anterior head-neck junction (line OS). The delta angle was the angle between lines ON and OT, which represented the concavity of the posterior head-neck junction.

The femoral neck-shaft relationship was evaluated with the neck-shaft angle and the neck version. In the AP photographs, the femoral neck-shaft angle was created by the intersection of the femoral neck axis and the femoral shaft axis. In the cephalocaudal photographs, the femoral neck version was the angle created by the intersection of the neck axis and the bicondylar plane, which was a line parallel to the table surface which both femoral condyles had been placed on. When the femoral neck was anteverted, this angle was located anterior to the bicondylar plane and was recorded as a positive number.

All of the measurements on the AP and cephalocaudal photographs were presented as means, standard deviations, ranges, and 95% confidence intervals. Statistical analysis was performed using SPSS Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Fifty-nine femora with an average age of 45.4 years at time of death were evaluated in this study. Forty-six femora (78%) were obtained from male skeletons, and 30 (51%) were right. The average femoral head diameter was 43.2 ± 3.6 mm (range: 35.5 to 50.7 mm). The results of the photographic measurements of the femoral head-neck relationships and neck-shaft relationships are in Table 1. The femoral head was located almost at the center of the femoral neck, which was demonstrated by the values of both the SOS/IOS and AOS/IOS ratios being close to 1. Both the AP and lateral physeal angles of the specimens were less than 90 degrees, so the femoral heads of this population were abducted and anteverted.

The femoral head-neck junctions presented their greatest concavity posteriorly because the average beta angle was lower than the average gamma, delta, and alpha angles. Regarding the parameters related to the cam morphology, 14 femora (24%) had an alpha angle greater than 50 degrees (Table 2), but all specimens had an anterior femoral head-neck offset ratio of more than 0.15. There were 7 femora (12%) that had a gamma angle greater than 50 degrees, but all specimens had a beta angle less than 50 degrees.

DISCUSSION

This study demonstrated that the femoral heads of Thai femora were located almost at the center of the femoral neck, with an abducted and anteverted rotational relationship to the neck axis. The femoral head-neck junctions demonstrated less concavity anteriorly and superiorly. Regarding the femoral neck-shaft morphology, the average femoral anteversion was 5.7 degrees, and the average neck-shaft angle was 129.5 degrees.

The values of the femoral head-neck translation in the present study was close to 1 for both the SOS/IOS and AOS/POS ratios. Toogood et al.,[3] evaluated the proximal femoral anatomy using digital photographic measurements of Caucasian and African-American femora. They demonstrated that the femoral head translated inferiorly with an average SOS/IOS ratio of 0.90, and anteriorly with an average AOS/IOS ratio of 1.14. The mean alpha angle was 45.6 degrees, which was comparable to the present study, but the mean gamma angle was 53.5 degrees, which was 7.9 degrees higher than that of the present study. This racial difference in the femoral head-neck morphology has also been demonstrated in the radiographic findings of the cam morphology of asymptomatic volunteers, which demonstrated a difference between Belgian and Chinese subjects.[17] This could be one of explanations for racial differences in the prevalence of hip osteoarthritis, which is much lower in the Asian population.[18]

The femoral head-neck rotation was evaluated by measuring the physeal scar orientation relative to the femoral neck axis. The results showed that the femoral head was rotated in abduction and anteversion, which could explain why there was less concavity of the femoral head-neck junction anteriorly and superiorly. Siebenrock et al.,[15] showed that there was greater lateral epiphyseal extension and less femoral head-neck offset at the anterosuperior quadrant in FAI patients than in control subjects.

Regarding the femoral neck-shaft relationship, the average neck-shaft angle in this study was 129.5

TABLE 1. Photographic measurements of the femoral head-neck and neck-shaft relationships.

Measurements	Mean (SD)	Range (Min, max)	95% CI (Lower, upper)
Head-neck translation			
SOS (mm.)	7.4 (1.3)	4.5, 11.2	7.0, 7.7
IOS (mm.)	7.4 (1.2)	5.0, 10.5	7.1, 7.7
SOS/IOS ratio	1.01 (0.21)	0.58, 1.53	0.96, 1.06
AOS (mm.)	10.0 (1.4)	7.1, 14.5	9.7, 10.4
POS (mm.)	10.4 (1.3)	7.2, 13.4	10.1, 10.8
AOS/POS ratio	0.97 (0.09)	0.74, 1.18	0.94, 0.99
Anterior head-neck offset ratio	0.23 (0.03)	0.17, 0.29	0.23, 0.24
Head-neck rotation			
AP physeal angle (°)	77.5 (11.0)	49.3, 105.4	74.6, 80.3
Lateral physeal angle (°)	81.9 (7.9)	64.7, 104.6	79.9, 83.1
Head-neck junction concavity			
Gamma angle (°)	45.6 (6.9)	37.6, 77.5	43.8, 47.4
Delta angle (°)	44.5 (3.4)	36.8, 52.7	43.7, 45.4
Alpha angle (°)	45.5 (11.1)	27.9, 80.2	42.6, 48.4
Beta angle (°)	36.2 (5.5)	25.1, 49.8	34.8, 37.7
Neck-shaft relationship			
Neck-shaft angle (°)	129.5 (5.1)	120.6, 141.9	128.2, 130.9
Neck version (°)	5.7 (7.8)	-12.6, 23.3	3.6, 7.7

Abbreviations: SOS = superior offset; IOS = inferior offset; AOS = anterior offset; POS = posterior offset; AP = anteroposterior

TABLE 2. Femoral head-neck junction concavity.

Measurements	< 50°	50° to 55°	> 55°
Gamma angle (n, %)	52 (88%)	2 (3%)	5 (8%)
Delta angle (n, %)	55 (93%)	4 (7%)	0
Alpha angle (n, %)	45 (76%)	2 (3%)	12 (20%)
Beta angle (n, %)	59 (100%)	0	0

degrees, which was comparable to the results reported by Toogood et al.,[3] but it was slightly higher than the previous three-dimensional measurement in computer aided design models of Thai subjects, which showed an average neck-shaft angle of 127.8 degrees.[11] In this study, the femoral neck orientation was 5.7 degrees of anteversion to the bicondylar plane, which was less than those found in previous studies, which reported values from 8.3 to 10.1 degrees of anteversion.[3,11,14]

There were some limitations. The higher proportion of male specimens might affect the values of the femoral head-neck morphology because the prevalence of cam morphology is usually male predominant.[5,8] There was no information on the height of the cadavers, although the average femoral head diameter in this study was 43 mm, which was similar to the mean value for 120 Thai volunteers in a study by Chantarapanich et al.[11]

In conclusion, the Thai femora had their heads located almost at the center of the femoral neck, and they were rotated in abduction and anteversion relative to the neck axis. The femoral head-neck junctions showed less concavity anteriorly and superiorly.

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Conflicts of interest: The authors declare that there are no conflicts of interest related to this study.

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Clinical and Laboratory Findings in Trauma-Induced Nail Dystrophy versus Onychomycosis

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ABSTRACT

Objective: The study aims to investigate prevalence, clinical characteristics, and laboratory findings of traumatic toenails in comparison with those of onychomycosis.

Methods: This cross-sectional study included hallux valgus (HV) patients attending Foot Clinic. Descriptive statistics, Chi-square test, Student's t-test and Mann-Whitney U test were used in statistical analysis.

Results: A total of 81 patients with a mean age of 65 years old, of which 77 were females (95.1%), were enrolled. The prevalence of onychomycosis was 25.9%, whereas that of trauma-induced nail dystrophy was 55.6%. There were 18 (22.2%) patients with fungal feet infection. Among 21 patients with nail infections, three had subclinical presentation. Fifteen patients (71.4%) with onychomycosis were infected with dermatophytes (DMPs). The others were caused by nondermatophytes (NDMs), of which 14.2% were *Neoscytalidium dimidiatum*. Comparing with traumatic nail, statistical analysis revealed HV angle of greater than 30 degrees and the presence of fungal foot infection to be significantly associated with onychomycosis ($p=.011$ and $p<.001$, respectively).

Conclusion: One-fourth of HV patients had onychomycosis. Severe foot deformities and concomitant fungal foot infection were significantly associated with onychomycosis. Holistic foot and nail examination is essential for distinguishing onychomycosis and traumatic induced nail dystrophy. Subclinical presentation was common.

Keywords: Onychomycosis; trauma-induced nail dystrophy; fungal foot infection; dermatophytosis; hallux valgus (Siriraj Med J 2018;70: 490-495)

INTRODUCTION

Trauma-induced changes in toenail units have been described in many previous reports.[1-7] The correlation between fungal infection and foot deformities was either lightly discussed or neglected altogether despite reports of foot trauma and deformity being predisposing factors for onychomycosis.[8] Most importantly, onychomycosis and trauma-induced nail dystrophy (traumatic toenail) are difficult to distinguish and may occur concomitantly. Hallux valgus (HV) was the most common foot deformity.[9] Thus, this study aimed to investigate prevalence, clinical characteristics, and laboratory findings relating to nail and foot abnormalities in patients with HV. A comparison between traumatic toenail and onychomycosis was also conducted.

MATERIALS AND METHODS

This was a cross-sectional study. Patients with HV who attended the Foot Clinic, Department of Rehabilitation Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University from January 2015 to August 2015 (study period) were included. The protocol for this study was approved

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by the Siriraj Institutional Review Board (Si 676/2014). Patients aged at least 18 years old were enrolled in this study and consent forms were obtained. Patients with underlying diseases known to cause nail abnormality (e.g., psoriasis) were excluded. Demographic data, family history, and predisposing factors for superficial fungal infection and HV were examined. Nail, foot, total skin examination and pedograph were performed by dermatologists and a physiatrist who specialized in foot disorders. Diagnosis and severity grading of hallux valgus were determined by angular deformity.[10] Hallux valgus angle was determined by measuring the relative position between the hallux and first metatarsal (in anthropometric terms) using the line between the most medial point of the heel and forefoot and the longitudinal axis of the big toe from weight bearing foot pressure graph (Fig 1). An angle greater than 14 degrees was interpreted to be hallux valgus, while those with a Staheli arch index of over 0.77 was defined as having flat feet.[11,12]

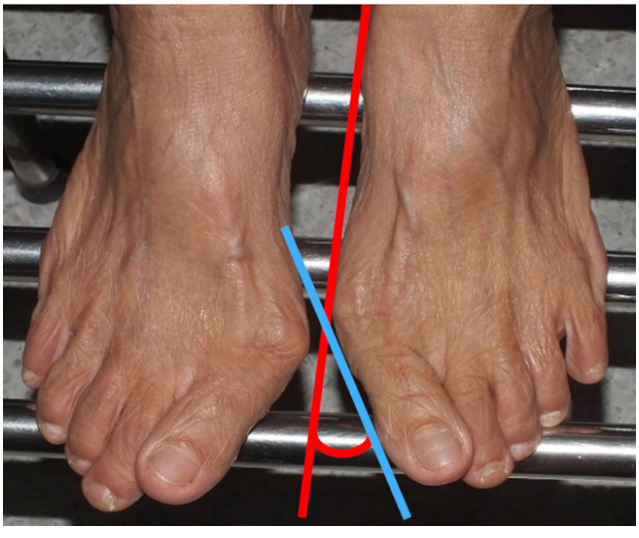


Fig 1. Hallux valgus angle
- Longitudinal axis of the big toe
- Line between the most medial point of the heel and forefoot

All toenails were evaluated for onychomycosis using Scoring Clinical Index for Onychomycosis (SCIO) score, which evaluates: (1) clinical forms of onychomycosis; (2) areas of involvement; (3) thickness of subungual hyperkeratosis; (4) locations of onychomycosis (fingernail or toenail, digit number); and, (5) age of patient. Higher SCIO score indicates increased likelihood of patients being positive for onychomycosis.[13] In each patient, nail sample was obtained from the nail with the highest SCIO score. Regarding foot involvement, specimen was collected from the foot with obvious scaly lesions. If all nails and both feet had a normal appearance, fungal culture and direct examination were performed on the right big toe and the right sole.

All nail and skin specimens were collected for microscopic examination in 20% potassium hydroxide (KOH) solution. For culture, each sample was inoculated in Sabouraud dextrose agar, with and without cyclohexylamine. Cultures were incubated at 27°C and examined every 4 days for 4 weeks. Identification of microorganisms was based on observations and descriptions of macroscopic and microscopic characteristics of colonies.

Diagnostic criteria according to a review by Gupta, *et al.*, were used for nondermatophyte molds (NDMs) onychomycosis diagnosis. Positive diagnosis was made if at least three of the following criteria were met: positive microscopic examination, positive mycological culture, at least two consistent isolations from repeated samplings, dermatophytes (DMPs) exclusion, and histology.[14] Patients with a normal clinical appearance and a positive microscopic examination and mycological culture for DMPs were diagnosed with subclinical presentation.

Statistical analysis

A sample size calculation was performed to determine the minimum requirement of a total sample size to detect the prevalence of fungal nail infection in hallux valgus patients. The prevalence of the infection (15.3%) found in Special Olympics athletes in Jenkins DW *et al.*,[15] was referenced as an anticipated population proportion. A minimum of 78 patients was necessary to provide 95% of confidence level and 0.08 of absolute precision required.

Descriptive statistics were used to describe demographic data, predisposing factors, clinical characteristics, and laboratory investigations. Descriptive data were presented as mean, median, minimum, maximum and percentages. Association between categorical variables was analyzed by v2-test or Fisher's exact test. Continuous variables with and without normal distribution were analyzed by Student's t-test and Mann-Whitney U test, respectively. A p-value ≤ .05 was considered statistically significant. All statistical analysis was performed using PASW for Windows version 18.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

A total of 81 patients with a mean age of 65 years old, of which 77 were females (95.1%), were enrolled. The prevalence of onychomycosis was 25.9%, whereas that of trauma-induced nail dystrophy was 55.6%. The remaining 15 nail specimens (20.6%) were of normal appearance and with normal fungal laboratory results. Among 21 patients with nail infection, three had subclinical presentation. Neither hematoma nor hemorrhage was

observed in any nails. Comparing with traumatic nail, statistical analysis revealed HV angle of greater than 30 degrees and the presence of fungal foot infection to be significantly associated with onychomycosis (p=.011 and p<.001, respectively). Clinical and laboratory findings were presented in Table 1 and Fig 2.

TABLE 1. Demographic data, predisposing factors for fungal infection and hallux valgus, clinical and laboratory presentations of nails in patients with hallux valgus

Factors	Onychomycosis (n=21)	Traumatic nail (n=45)	P-value
Sex; female	21 (100%)	41 (91.1%)	0.901
Mean age (SD; year)	67.4 (9.27)	65.9 (7.59)	0.477
Agricultural work	7 (33.3%)	13 (28.9%)	0.714
Walking barefoot	7 (33.3%)	8 (17.8%)	0.210
Poor circulation	4 (19%)	5 (11.1%)	0.450
Diabetes mellitus	4 (19%)	8 (17.8%)	1
Family history of superficial fungal infection	3 (14.3%)	6 (13.3%)	1
History of pedicure	8 (38.1%)	15 (33.3%)	0.705
Plantar hyperhidrosis	7 (33.3%)	14 (31.1%)	0.857
Family history of HV	9 (42.9%)	25 (55.6%)	0.336
Foot trauma and/or improper shoe	8 (38.1%)	18 (40%)	0.883
Median duration of HV (years)	20 (1,40)	10 (0.8, 75)	0.241
Flat feet	12 (63.2%)	19 (42.2%)	0.126
Overriding toe	11(52.4%)	13 (28.9%)	0.065
Presence of callus	17 (81%)	40 (88.9%)	0.450
Clinical nail characteristics			
Normal appearance	3 (14.35%)	0	-
Nail bed keratosis	14 (66.7%)	28 (62.2%)	0.727
Onycholysis	2 (9.5%)	7 (15.6%)	0.707
Abnormality of nail curvature	11 (52.4%)	22 (48.9%)	0.792
Discoloration	18 (85.7%)	37 (82.2%)	1
Site of toenail:			
Big toenail	8 (38.1%)	23 (51.1%)	0.324
Unilateral involvement	13 (61.9%)	36 (80%)	0.117
SCIO index (mean ± SD)	9.3 (7.69)	9.6 (7.79)	0.860
HV with angle greater than 30 degrees	18 (85.7%)	24 (53.3%)	0.011*
Fungal foot infection	14 (66.7%)	5 (11.1%)	<0.001*

*Statistical significance; p≤.05

Abbreviations: OR = odds ratio; CI = confidence interval; SD = standard deviation; SCIO = Scoring Clinical Index for Onychomycosis



Fig 2. Representative nail images of patients with hallux valgus: (a) dermatophyte onychomycosis; (b) nondermatophyte onychomycosis; (c) traumatic toenail; (d) subclinical onychomycosis.

Of 21 cases with onychomycosis, 15 (71.4%) were infected with DMPs. Ten were *Trichophyton mentagrophytes* and four were *Trichophyton rubrum*. Six nails were infected with nondermatophytes (NDMs), of which three (14.2%) were caused by *Neoscytalidium dimidiatum*.

Regarding investigation of 81 feet, fungal infection was found in 18 (22.2%). For clinical presentation, 15 feet were moccasin-type, one foot was vesicular-type, and one foot was interdigital-type. Subclinical presentation was observed in three feet. Concerning causative agents, 17 (94.5%) were infected with DMPs. Fourteen were *T. mentagrophytes*, two were *T. rubrum*, and one was infected with *N. dimidiatum*.

DISCUSSION

Foot trauma and shoe friction due to asymmetric gait and foot deformity were found to cause toenail changes.[2,4,6,7] Asymmetric gait toenail unit syndrome (AGNUS) was reported to be the most common worldwide toenail abnormality in shoe-wearing societies and may be responsible for 70-73% of abnormal toe nails.[7] Previous studies reported the prevalence of traumatic toenail to be approximately 14.2% in the normal population.[3] Regarding patients with HV in the present study, the prevalence of trauma-induced toenail dystrophy was higher (55.6%) due to the effect of foot deformities. With regard to nail bed keratosis, abnormality of nail curvature, and onycholysis caused by pressure from shoes, these abnormalities usually started from the medial aspect of the nail, with unilateral side involvement then gradually progressing to lateral aspect and bilateral sides. Hemorrhage or hematoma may be found in traumatic onycholysis.[1,3,4] However, in this study, neither hemorrhage nor hematoma was observed in patients with HV. Early recognition of traumatic toenail is essential for avoiding or minimizing nail abnormalities.

Foot and nail traumas and/or deformities were found to be important predisposing factors for onychomycosis.[6,8] Onychomycosis and traumatic toenails are often not clinically distinguishable. Nail bed keratosis, onycholysis, and abnormality of nail curvature can be found in both conditions. Furthermore, onychomycosis and traumatic toenail may occur concomitantly.[1] In the present study, there were two clinical characteristics that were significantly related to onychomycosis. First, the presence of fungal foot infection was meaningfully associated with onychomycosis. Many previous studies have supported association between fungal foot infection and onychomycosis. The spread of infection to adjacent tissues should raise awareness of the need for whole skin examination in patients with fungal infection.[3,6,14,16,17] Second, HV angle of greater than 30 degrees was associated with fungal infection. Patients with severe hallux valgus typically presented with overriding toes, particularly the first and second toes. In patients with overriding toes, fungal infection was normally observed in the moist inter-web spaces. These clinical findings may help clinicians identify patients potentially prone to fungal infection. However, mycological laboratory investigation was required for a definite diagnosis, and decisions regarding antifungal therapy, and evaluation of mycological cure. Correction of the deformity will be helpful to reduce risk of infection. While patients are waiting for a surgical correction, treatment for superficial fungal infection is beneficial to prevent widespread infection and superimposed bacterial infections.

Notably, a normal appearance did not indicate that the patient was free of fungal infection. Subclinical infection was frequently found. Previous studies reported 17.0% and 1.5% of population with and without tinea pedis had subclinical onychomycosis as proven by nail histology.[16] Similar to this study, the prevalence of subclinical onychomycosis was 14.3%. Among those with foot infection, 16.7% also had subclinical foot infection. Subclinical infection was important for detecting reservoir of fungus that could lead to spread of infection to adjacent skin and nail. Subclinical infection can also cause reinfection if treatment is not appropriate. For example, topical antifungal agents that were normally applied to treat tinea pedis did not affect onychomycosis. The use of oral antifungal therapies in patients with recurrent tinea pedis in the treatment of subclinical nail infection requires further study. However, holistic skin and nail examination in patients with tinea pedis or onychomycosis was absolutely necessary. Laboratory investigation should be considered in patients with clinical suspicion of superficial fungal infection.

Nail changes are not always caused by fungus or trauma. Many diseases appear similar to onychomycosis, such as psoriasis and lichen planus. It is advisable to perform laboratory investigation for a definitive diagnosis of fungal infection before initiating any antifungal therapy. Direct examination by KOH preparation and fungal culture remain gold standard methods for diagnosis. Misdiagnosed onychomycosis may lead to unnecessary use of systemic antifungal agents, resulting in medication-related side effects and no improvement in condition. Patients with onychomycosis may also develop concomitant traumatic toenails. It is for this reason that clinical cure may not look completely normal, even though the patient received effective antifungal therapy.[1,2] Once again, mycological examination played a critical role in evaluating mycological cure and completion of treatment.

Regarding NDMs infection in traumatic toenails, colonization or true pathogen is still under debate. NDMs onychomycosis in this study was diagnosed according to a minimum of three of the following criteria: positive microscopic examination, positive mycological culture, at least two consistent isolations from repeated samplings, and DMPs exclusion.[14] Histology was not performed in this study to confirm nail involvement. One third of NDMs onychomycosis patients had foot infection with the same NDMs agents, which may be transmitted from the ground and may serve as a reservoir for spreading infection to nails. Moreover, alteration of toenail unit anatomy caused by onycholysis, nail bed keratosis, and superficial nail plate damage in patients with AGNUS and foot deformities were essential predisposing factors for NDMs onychomycosis.[6] Therefore, NDMs onychomycosis in this study may be the true pathogens. Follow-up clinical and laboratory findings to evaluate improvement after treatment may be another key to determining the true pathogen.

Several limitations were present in this study. First, the majority of patients were female. This may be due to the fact that hallux valgus was found predominantly in female. Moreover, female patients were more concerned about their feet and thus more enthusiastic to participate in the study. Second, this study focused only on patients with HV, which has been the most common foot deformity. Further studies may be conducted with patients with other types of foot deformities and/or those without foot deformities.

CONCLUSION

In conclusion, this study revealed that one-fourth of HV patients had onychomycosis. Severe foot deformities and concomitant fungal foot infection were significantly associated with onychomycosis. Holistic foot and nail examination is essential to distinguish onychomycosis and traumatic induced nail dystrophy. Subclinical presentation was common.

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Factors Associated with Learning Outcomes in First Pre-clinical Year Medical Students at the Faculty of Medicine Siriraj Hospital, Mahidol University

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ABSTRACT

Objective: Electronic learning system (e-lecture) has been developed and used as a tool to assist students' learning. Usage of e-lecture and learning behaviors were evaluated for their associations with learning outcomes.

Methods: This questionnaire-based, cross-sectional study enrolled 107 first pre-clinical year medical students at the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Information on learning outcomes of biomedical subjects and learning behavior including e-lecture use, self-study time, skipping class and inattention were collected. Mann-Whitney U test and logistic regression were used for statistical analysis.

Results: Compared to students who earned average biomedical grades of >3.0, students who earned average grades ≤3.0 significantly used more e-lecture (median, IQR 63.81, 49.17-70.03 and 31.08, 11.29-51.49, respectively, p =0.001), had more inattention time during lectures (median, IQR, 22.13, 14.94-31.19, and 13.1, 8.05-20.30, respectively, p=0.008), and spent less time for self-study and review of lessons (median, IQR 102.00, 68.00-176.50, and 147.50, 106.25-246.00, respectively, p=0.04). For each one hour increase in e-lecture usage, the chance of earning an average biomedical grade of >3.0 was decreased by 4%. Positive correlations were found between e-lecture usage with skipping class and with inattention during lecture (coefficients = 0.31 and 0.37, with p=0.001 and p<0.001, respectively). Time spent for self-study and review of lessons negatively correlated with inattention during lectures (coefficient =-0.28, p=0.003).

Conclusion: E-lecture may be misused by students who have poor learning behaviors as a substitute for in-class lectures. Time voluntarily spent in e-lecture may be an indicator for students who need educational guidance and/or counseling.

Keywords: Learning outcomes; learning behavior; electronic-lecture system; e-lecture; medical education; medical student (Siriraj Med J 2018;70: 496-501)

INTRODUCTION

Identifying new methods for improving learning outcomes in undergraduate medical students is an important challenge. Electronic learning tools (e-learning) designed to support students have developed in popularity over the last decade.[1-3] Benefits of e-learning include convenient access and compatible with lifestyles.[4-11] However, the efficacy in imparting knowledge is still being debated.[12] One meta-analysis compared traditional and internet-based learning in health profession and found the two methods to be equal in learning outcomes.[13] Another study on learning outcomes in *Gross Anatomy* concluded that

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students could decrease time and improve satisfaction using supplemental videos, despite a failure to improve final learning outcomes.[14]

The Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand ("the faculty") offers a six-year undergraduate medical degree program. Lectures for pre-clinical students were traditional one-way lectures. First pre-clinical year students are required to attend at least 80% of classes in order to be eligible for evaluations. The faculty supports and provides self-learning tools including libraries and e-learning systems with unlimited access. E-learning materials include videos of previous lectures, training materials, self-assessment tests, and others. For students in their first pre-clinical year, video recorded lectures (e-lecture) comprise the majority of e-learning resources.

In the real world, many learning behaviors besides electronic learning system usage also affect learning outcomes.[15-18] Absence from class was related to negative academic achievement and positive achievement was related to reading to a level of expectation.[19-20] While several studies have investigated the effect of learning behaviors on academic achievement, no studies have explored the association between learning outcomes and voluntary use of e-learning combined with individual learning behaviors in first pre-clinical year medical students. The objective of this study was to evaluate the association between learning behaviors and learning outcomes in the first pre-clinical year medical students.

METERIALS AND METHODS

This was a questionnaire-based cross-sectional study approved by Siriraj Institutional Review Board (Si 267/2013). Eligible students must have been enrolled in and passed all first pre-clinical year courses in the academic year 2012. Recruitment of participants was conducted in a lecture class. Students were informed and asked to join the study voluntarily. Those who completed and returned the questionnaire were considered to have provided informed consent.

The Thai language self-reported questionnaire was designed to evaluate following parameters: 1) grade of each biomedical subject taken in the first pre-clinical year; 2) time voluntarily spent using e-lecture; 3) time spent for self-study and review of lessons in three-week period before examinations not including e-lecture; 4) time skipping lecture classes, defined as not being present for >50% of a scheduled class period; and, 5) inattention time in class (e.g., napping, daydreaming, using mobile electronic device, and/or engaging in other non-educational activities).

Each questionnaire item was reviewed and edited by five medical students who did not take part in conducting this study. Questionnaires were provided to all 324 first pre-clinical year medical students enrolled for the 2012 academic year.

The dependent variables were biomedical grades earned in the first pre-clinical year including *Gross Anatomy I and II, Histology, Neuroanatomy, Embryology, Physiology, Biochemistry, Preventive Medicine, Medical Education & Medical Profession, and Health Promotion and Humanistic Medicine*. The grades, A, B+, B, C+, C, D+, and D, were transformed to numerical data (4.0, 3.5, 3.0, 2.5, 2.0, 1.5, and 1.0, respectively) for analysis. Time voluntarily spent in behaviors of interest including skipping classes and inattention during lecture were estimated by using a percentage replied in the questionnaire for each subject multiplied by scheduled and additional lectures duration in the courses and reported in hours.

GRADE was defined as a credit-weighted average academic grade for all the previously described biomedical subjects. Others grades were excluded.

E-LECTURE was defined as a summation of time spent in e-lecture for every subject. STUDY was defined as a summation of time spent in self-study and review of lessons in three-week period before examinations. SKIP was defined as a summation of time skipping traditional lecture classes. INATTENTION was defined as a summation of inattention time in lecture.

Participants were separated into two groups according to GRADE, HIGH-GRADE for GRADE >3.00 and LOW-GRADE for GRADE ≤3.00. Kolmogorov-Smirnov test revealed a non-normal distribution of the data. The data were described as median (interquartile range) or proportion, as appropriate. Mann-Whitney U test was used to compare learning behaviors between the two study groups. Multiple logistic regression analysis was employed to adjust for confounding effects. Correlation coefficients were calculated to explore collinearity among the variables.

RESULTS

Eight responses were excluded due to missing data and 107 participants, 55.14% male and 44.86% female, were included. The response rate was 33.02%. Data were analyzed using STATA 13.0 (StataCorp, Texas, USA). There was no statistically significant difference in GRADE between male and female (p = 0.81). The median age (range) was 20 (19-22) years.

The subjects with the highest median percentage of time voluntarily spent in e-lecture were *Physiology* (60.00%), *Biochemistry* (50.00%), *Neuroanatomy* (30.00%),

and *Preventive Medicine* (30.00%). The subjects that had the highest median time spent in self-study were *Gross Anatomy II* (28.00 hours), *Gross Anatomy I* (24.00 hours), *Neuroanatomy* (20.00 hours), and *Physiology* (20.00 hours). The subject that had the highest median percentage of inattention time in lecture classes was *Health Promotion and Humanistic Medicine* (30.00%). The subjects with the highest proportion of students skipping class were *Health Promotion and Humanistic Medicine* (46.73%), *Preventive Medicine* (28.04%), and *Medical Education & Medical Profession* (26.17%). The median GRADE among participants was 3.58 (IQR = 0.63). Other descriptive results are shown in Table 1.

Table 2 demonstrated learning behaviors of HIGH-GRADE (n=92) and LOW-GRADE (n=15) students. Time spent in e-lecture in LOW-GRADE was significantly higher than in HIGH-GRADE (median 63.81, IQR 49.17-70.03 and median 31.08, IQR 11.29-51.49, respectively, p =0.001). Inattention in LOW-GRADE was significantly higher than in HIGH-GRADE (median 22.13, IQR 14.94-31.19, and median 13.1, IQR 8.05-20.30, respectively, p=0.008). In contrast, self-study in LOW-GRADE was significantly lower than in HIGH-GRADE (median 102.00, IQR 68.00-176.50, and median 147.50, IQR 106.25-246.00, respectively, p=0.04).

TABLE 1. Learning outcome and learning behaviors of study participants (n=107)

Variables	Median	IQR[1]	Maximum	Minimum
Grade[2]	3.58	3.23 – 3.87	4.00	2.00
E-lecture[3] (hour)	35.64	13.56 – 55.80	100.00	0.00
Self-study[4] (hour)	143.00	101.00 – 221.00	528.00	0.00
Skip[5] (hour)	0.14	0.00 – 3.15	73.48	0.00
Inattention[6] (hour)	13.65	8.15 – 21.99	108.57	0.00

[1]Interquartile range
[2]Credit-weighted average grade
[3]Summation of time spent in the E-lecture system
[4]Summation of the time spent in self-study and review of lessons in the three weeks before examinations in each subject (not including time voluntarily spent in the E-lecture system)
[5]Summation of time skipping lecture classes
[6]Summation of inattention time in lecture classes.

TABLE 2. Difference in grade and learning behaviors between low-grade[1] (n=15) and high-grade[2] (n=92) groups

Variables	Low-Grade[1] (median, IQR[3])		High-Grade[2] (median, IQR[3])		P-value
Grade[4]	2.63	2.48 – 2.88	3.70	3.43 – 3.89	<0.000*
E-lecture[5]	63.81	49.17 – 70.03	31.08	11.29 – 51.49	0.001*
Self-study[6]	102.00	68.00 – 176.50	147.50	106.25 – 246.00	0.040*
Skip[7]	2.04	0.03 – 5.93	0.06	0.00 – 2.38	0.055
Inattention[8]	22.13	14.94 – 31.19	13.11	8.05 – 20.30	0.008*

*p-value less than 0.05 indicates statistical significance
[1]Participants who earned a credit-weighted average grade equal to or below 3.00
[2]Participants who earned a credit-weighted average grade above 3.00
[3]Interquartile range
[4]Credit-weighted average grade
[5]Summation of time spent in the E-lecture system (hour)
[6]Summation of the time spent in self-study and review of lessons in the three weeks before examinations in each subject (not including time voluntarily spent in the E-lecture system (hour)
[7]Summation of time skipping lecture classes (hour)
[8]Summation of inattention time in lecture classes. (hour)

Relationships between GRADE and all independent variables were analyzed by logistic regression. Univariate analysis showed that E-LECTURE and INATTENTION significantly decreased the chance to be in HIGH-GRADE (ORs 0.95, 95%CI 0.93 to 0.98 and 0.95, 95%CI 0.91 to 0.99, respectively). Multiple logistic regression was performed. In model 1, all independent variables were included, but in model 2, only variables with significant associations (p <0.05) in univariate analysis, E-LECTURE and INATTENTION, were used. Only E-LECTURE remained significantly associated with decreasing the chance to be in HIGH-GRADE in both models (OR 0.96, 95%CI 0.93 to 0.99). The results of logistic regression analysis were as shown in Table 3.

Multicollinearity was evaluated by Spearman's correlation coefficients for all learning behaviors. Statistically significant positive correlations were identified between E-LECTURE with SKIP, and E-LECTURE with INATTENTION (coefficients = 0.31 and 0.37, with p=0.001 and p<0.001, respectively). Moreover, SELF-STUDY was found to have significant negative correlation with INATTENTION (correlation coefficient = -0.28 with p=0.003).

DISCUSSION

This study found that time voluntarily spent in the e-lecture system was significantly associated with poor learning outcomes. This finding was not consistent with those of prior studies.[4,5,14] The e-lecture system was established by the faculty to support students in self-directed learning. The intended use of the system was as supplemental support to enhance learning outcomes. Rather, this study found a voluntary use of e-lecture to be statistically significantly associated with skipping class and inattention in the classroom which imply that students who skipped classes more often and lacked in-class attention tended to use the e-lecture to compensate. This finding corresponds with a prior study found that students relying on video lecture records tended to get lower scores in *Histology* than who regularly attended class.[21]

In this study, learning outcomes was positively correlated with time spent in self-study and review of lessons before examinations, but was negatively correlated with duration of inattention in classes. An earlier study reported that percentage of reading to a level of expectation had a positive effect on learning outcomes in *Physiology, Anatomy*, and *Biochemistry*.[20] The cognitive load theory[22] stated that attention is essential for long-lasting knowledge to be created from incoming information also supports this finding.

This study found that many learning behaviors,

TABLE 3. Associations of learning behaviors with grade[#]

Learning Behaviors	ORs ± Std.Error	95% CI	P-value
Univariate Logistic Regression			
E-lecture[1]	0.95 ± 0.01	0.93 to 0.98	0.001*
Self-study[2]	1.01 ± 0.00	1.00 to 1.01	0.067
Skip[3]	0.96 ± 0.02	0.94 to 1.01	0.530
Inattention[4]	0.95 ± 0.02	0.91 to 0.99	0.017*
Multivariate Logistic Regression			
Model 1: E-lecture[1*]	0.96 ± 0.02	0.93 to 0.99	0.008*
Model 2: E-lecture[1**]	0.96 ± 0.01	0.93 to 0.99	0.005*

*p-value less than 0.05 indicates statistical significance
[#]Credit-weighted average grade of all subjects
[1]Summation of time spent in the E-lecture system
[2]Summation of the time spent in self-study and review of lessons in the three weeks before examinations in each subject (not including time voluntarily spent in the E-lecture system)
[3]Summation of time skipping lecture classes
[4]Summation of inattention time in lecture classes.
*All independent variables included in multivariate model (1, 2, 3, and 4)
**Independent variables with statistical significance at P <0.05 in univariate analysis included in multivariate model (1 and 4)

but not all, affect learning outcomes. As such, students may improve their learning outcomes by developing personal strategies and teachers may be able to use these behaviors as indicators for early detection of learning problems.

The electronic learning system is an essential and effective educational tool. Students prefer it because of advantages that a traditional lecture cannot provide, such as flexibility and an improved ability to accommodate individual learning styles.[23-25] However, there are some disadvantages.[4,24,25] Traditional lectures allow students to directly interact with the instructor.[10] The e-lecture is similar to the traditional lecture system in that it is communication which may be not a perfect tool for replacing in-class lectures. Time voluntarily spent in the e-lecture could potentially be a red flag for identifying students who need educational guidance. Administrators and/or lecturers may consider monitoring amount of time each student spends in e-lecture and evaluate if a student is exhibiting signs of possibly needing help.

We also found that *Health Promotion and Humanistic Medicine, Preventive Medicine, Medical Education & Medical Profession* had the highest proportion of students skipping class. Possible causes may be explained by students' perceptions and nature of these subjects. Medical students give priority to the subjects related to pure biomedical science, since they think that only knowledge in this area is enough for being doctors. These subjects involved the knowledge in the field of public health, social sciences, and other "soft skills" which students are not familiar with and do not appreciate the importance of these as a part of being good doctors. From our experience, some students have no motivation, or cannot relate these subjects with patient care or medical practice at all. Promoting self-motivation or delivering knowledge using student-centered approach may be solutions to these problems.

This study had some limitations. A cross-sectional design does not allow demonstration of temporal relationships. Responder and recall biases may have occurred. Other limitations include the response rate which was only 33.02%., the size of the LOW-GRADE group (n=15) was much smaller than the size of the HIGH-GRADE group (n=92). Lastly, the average biomedical GPA who agreed to participate was 3.6, it is likely that selection bias may have occurred. Students with low GPAs may have demurred and decided against participating in this study. A future study to confirm this finding should be performed.

Multicollinearity was found among the following learning behavior variables: skipping class, inattention in class, and e-lecture usage. The root causes of these behaviors should be explored. Achievement motivation may be the cause of some behaviors. One study reported that achievement motivation could predict academic achievement in health sciences students.[26] Future studies should be undertaken to explore associations between other factors and learning outcomes and endeavor to identify associations among that broadened list of learning behaviors.

An increase in usage of the e-lecture system was significantly associated with the likelihood of having low learning outcomes. In addition, more e-lecture usage was associated with higher rates of skipping class and inattention in class. The e-lecture system, like in-class lectures, delivers one-way information and should not be used as an alternative to in-class lectures. Learning outcomes were positively associated with time spent in self-study and review of lessons. Based on these findings, lecturers and/or administrators should consider monitoring student use of the electronic learning system to observe for behaviors that may suggest learning-related problems and/or to identify students who may require support. These findings may also benefit students by helping them to identify potential misdirection and motivate them to apply learning strategies improving learning achievement.

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Normal Adrenal Glands in Multidetector Computed Tomography

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ABSTRACT

Objective: To characterize normal adrenal glands in an adult Thai population using multidetector computed tomography (MDCT).

Methods: We retrospectively reviewed the scans of 175 consecutive patients who underwent renal CT angiography for potential kidney donation with attention to the location, morphology, and enhancement of both adrenal glands. Each reader measured the body and limb thickness in the axial and coronal planes, and the length in the axial plane. The interclass correlation coefficient (ICC) between the two readers was generated. We employed mean, SD, and 95th percentile of normal adrenal gland size by using pooled data of both reviewers.

Results: The 95th percentiles of normal adrenal glands, which might be useful as cutoff values to distinguish between normal and pathological adrenal gland size, were calculated. The right adrenal gland was more often relatively cephalad to the left adrenal (58.9%). The most common morphology of both adrenal glands was the inverted Y, with inverted V and linear shapes less common. Both adrenal glands showed homogeneous enhancement (100%) in the arterial and nephrographic phases.

Conclusion: The majority of normal adrenal glands were right relatively cephalad to left, inverted Y shape, and homogenous enhancement, using the 95th percentile of size normal adrenal gland as a cutoff value to differentiate between normal and thickened limbs and body.

Keywords: Size; morphology; adrenal gland; multidetector computed tomography (Siriraj Med J 2018;70: 502-506)

INTRODUCTION

Recent advances in high-resolution thin-slice multidetector computed tomography (MDCT), and the widespread use of MDCT in abdominal imaging, has led to increased detection of adrenal pathology, such as focal nodules and diffuse enlargement. The incidence of adrenal nodules is approximately 3-7% in the adult population.[1,2] The vast majority of adrenal nodules in patients without known malignancy are benign adenomas.[3,4] The incidence of adrenal nodules is slightly increased about 9-13% in oncologic patients.[5,6] Therefore, the adrenal gland is a common site for metastases, particularly those arising from lung, breast, lymphoma, gastrointestinal tract, thyroid, kidneys and melanoma. However, only 26-36% of adrenal nodules are metastases.[5] Furthermore, diffuse enlargement is another common feature of adrenal gland pathology and the causes include bilateral adrenal hyperplasia (BAH), lymphoma, metastatic disease, tuberculosis, and histoplasmosis.

CT has been the primary imaging modality in the

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detection and characterization of adrenal lesions. To better recognize pathology, basic knowledge of normal size and morphology of the adrenal glands is important, particularly with small nodules and subtle lesions. Previous studies[7-10] described the normal size and morphology of the adrenal glands worldwide. However, there is a lack of data about normal size and morphology of adrenal glands in adult Thai population by using MDCT. Moreover, BAH is commonly observed in primary aldosteronism. In BAH, both adrenal glands are usually enlarged and the mean adrenal limb width is greater than normal. In general, the normal limits of adrenal gland size usually relied on the data of Vincent et al.,[11] and Lingam et al.,[12]; although we found some limitations in their studies including small number of patients.

The purpose of our study was to determine the size of normal adrenal glands, using the 95th percentile as a cutoff value, and to evaluate the location, morphology, and enhancement pattern of normal adrenal glands in adult Thai population using MDCT.

MATERIALS AND METHODS

Patient selection

The approval for this retrospective study was obtained by the ethical committee of the institute (Si 106/2013). A total of 175 consecutive adult patients between January 2006 and December 2013 underwent renal CT angiography for potential kidney donation in our institute. The exclusion criteria of the study were:

1. Patients who had obvious abnormal adrenal gland pathology, such as mass, cyst, or calcifications
2. Patients with previous history of abdominal trauma
3. Patients with history of tuberculosis or other granulomatous infections
4. Patients with a history of coagulopathy, taking anticoagulants, or any herbal medication

The 175 patients' (65 men and 110 women, mean age 35 years ± 9.0 SD, range 19-56 years) histories were reviewed by a resident using the medical records, medical record archiving system, and laboratory report system. We did not find any excluded patient in this population.

The CT technique

All examinations were performed with 64-detector MDCT scanners (Somatom Definition, Siemens Medical Solutions Erlangen, Germany and Light Speed VCT, General Electric Medical Systems Milwaukee WI, USA). The scans were performed with the following sequences: 1) scanogram (scout), 2) axial precontrast phase (images were obtained from hepatic dome to pubic symphysis), axial arterial phase (timed using a region of interest placed at the proximal renal artery, from hepatic dome to iliac crest), 3) axial nephrographic phase (delayed 100 seconds after contrast medium injection obtained from hepatic dome to pubic symphysis), 4) axial excretory phase (delayed 10 minutes after contrast medium injection from hepatic dome to pubic symphysis). The CT parameters included: 120 kVp voltage, 500 mA (GE) or 250 mA with CARE Dose 4D® in order to reduce radiation (Siemens) current, a gantry rotation time of 0.5 second, section thickness of 1.25-1.5 mm and a pitch of 1:0.9 (Siemens) or 1: 0.984 (GE).

Imaging analysis

Two experienced radiologists (W.T. and V.S., with 7 and 4 years of experience in abdominal imaging, respectively), interpreted the CT images of the adrenal glands at picture archiving and communication systems workstations (PACS; Synapse, Fujifilm Medical Systems, Tokyo, Japan). Each reader independently assessed the images and any disagreements were subjected to consensus. The location (cephalad and caudal relative to contralateral adrenal gland), morphology of each gland (linear, inverted V, inverted Y, and triangular shapes) and enhancement (homogeneous or heterogeneous enhancement, were evaluated in arterial and nephrographic phases). Each reader measured the thickness of the body (defined as the maximum thickness of the body perpendicular to the long axis in axial and coronal views [measured only in inverted V, inverted Y and triangular shape]) and the width of both limbs (defined as the maximum width of each limb in the axial and coronal planes [measured only in inverted V, inverted Y and linear shape]) the length of the glands (defined as the longest length from tip to the longest limb of the adrenal gland in the axial plane) using electronic calipers.

Statistical analysis

The location, morphology, and enhancement pattern of both adrenal glands measured by each reader were determined in terms of percentage. The size (including thickness, width and length) of both adrenal glands were determined in terms of mean, range, standard deviation (SD) and the 95th percentile (equal to mean + 1.96 SD). The interclass correlation coefficient (ICC) in measurement of size of both adrenal glands between the two readers was generated. An ICC value < 0.4 indicated poor inter-rater reliability; ≥ 0.4 < 0.75 which indicated fair or good inter-rater reliability, and ≥ 0.75 indicated excellent inter-rater reliability. The 95th percentile of normal adrenal gland size was determined as the cutoff value. Statistical analysis was performed using SPSS version 15.0 for Windows (IBM, Chicago IL, USA).

RESULTS

Size of adrenal glands

Table 1 shows the size of both adrenal glands measured by both readers (W.T. and V.S.). The ICCs in measurement of thickness, width, and length of both adrenal glands were about 0.3-0.4, 0.25-0.5, and 0.6-0.8, respectively. Furthermore, we evaluated the mean, SD, and 95th percentile of thickness, width and length of both adrenal glands by using pooled data of both reviewers.

Location of adrenal glands

The location of the right adrenal gland was more commonly cephalad to the left gland (58.9%).

Morphology of adrenal glands

The most common morphology of both adrenal glands was the inverted Y (85.8% on the right side and 93.7% on the left side). The less common morphologies of the adrenal glands were inverted V and linear shapes. The morphologies are shown in Table 2 and Fig 1.

TABLE 1. Measurement size of both adrenal glands.

	Parameters	Reader 1 (W.T)				Reader 2 (V.S)				Both readers
		Mean	Range	SD	P 95	Mean	Range	SD	P 95	Mean±SD (P 95)
Right adrenal gland	Axial thickness	4.91 mm.	2.8-6.75	0.72	6.04	4.52 mm.	2.33-6.73	0.87	6.04	4.7±0.64 (5.9)
	Coronal thickness	4.50 mm.	2.1-14.0	1.2	6.3	3.73 mm.	2.05-6.05	0.94	5.46	4.1±0.86 (5.5)
	Axial width medial limb	3.98 mm	2.53-6.75	0.68	5.14	3.27 mm.	1.43-5.32	0.77	4.66	3.6±0.62 (4.6)
	Coronal width medial limb	4.20 mm.	2.13-14.0	1.1	5.57	3.31 mm.	1.41-6.39	0.80	4.68	3.7±0.77 (4.8)
	Axial width lateral limb	3.83 mm.	2.12-6.75	0.81	5.13	3.05 mm.	1.48-5.65	0.78	4.55	3.4±0.66 (4.6)
	Coronal width lateral limb	3.68 mm.	1.9-14.0	1.14	5.08	2.72 mm.	1.28-5.72	0.78	4.17	3.2±0.75 (4.3)
	Axial length	3.32 cm.	2.36-4.8	0.42	4.00	3.14 cm.	1.44-5.20	0.58	4.08	3.2±0.44 (4.0)
Left adrenal gland	Axial thickness	5.03 mm.	2.53-12.4	1.27	6.90	4.45 mm.	2.39-7.79	1.06	6.48	4.7±0.97 (6.2)
	Coronal thickness	4.69 mm.	2.76-11.34	1.12	6.62	4.11 mm.	1.98-8.33	1.06	5.88	4.4±0.9 (6.0)
	Axial width medial limb	5.00 mm.	3.2-7.8	0.9	6.6	4.14 mm.	2.26-7.23	0.84	5.54	4.6±0.7 (5.7)
	Coronal width medial limb	4.88 mm.	3.04-7.11	0.87	6.28	4.06 mm.	1.88-9.45	1.05	5.68	4.5±0.74 (5.7)
	Axial width lateral limb	4.05 mm.	1.98-6.34	0.87	5.40	3.26 mm.	1.15-5.85	0.88	4.74	3.6±0.7 (4.8)
	Coronal width lateral limb	3.81 mm.	2.22-6.79	0.90	5.47	3.04 mm.	1.40-7.62	0.93	4.87	3.4±0.7 (4.6)
	Axial length	2.99 cm.	1.32-5.79	0.70	4.30	2.70 cm.	1.23-5.37	0.74	4.21	2.8±0.7 (4.2)

Abbreviations: P 95 = 95th percentile, SD = standard deviation

TABLE 2. Morphology of both adrenal glands.

Adrenal gland	Morphology/Shape	Number (%)
Right adrenal gland	Linear	2/175 (1.1%)
	Inverted V	23/175 (13.1%)
	Inverted Y	150/175 (85.8%)
	Triangular	0/175 (0%)
Left adrenal gland	Linear	1/175 (0.6%)
	Inverted V	10/175 (5.7%)
	Inverted Y	164/175 (93.7%)
	Triangular	0/175 (0%)



Fig 1. The morphology of normal adrenal glands.
A. Portovenous phase of CT shown inverted Y shape of left adrenal gland.
B. Portovenous phase of CT shown inverted V shape of right adrenal gland.
C. Portovenous phase of CT shown linear shape of left adrenal gland.

Pattern of enhancement

All glands showed homogeneous enhancement (100%) in the arterial and nephrographic phases.

DISCUSSION

Improvement in CT scanner technology increases the ability to demonstrate normal and abnormal adrenal glands. In our study, using 64-detector MDCT in all CT examinations with 1.25–1.5 mm. slice thickness, all normal adrenal glands were clearly identified. Our study was performed in potential renal donors that were representative of the normal population and we used a larger number of patients (175 patients) than the previous studies.[11,12] Additionally, our study performed thin-slice CT imaging which is more accurate. Our study found poor-to-fair interrater reliability of ICC in measurement of the thickness and width of both adrenal glands and fair-to-excellent inter-rater reliability of ICC in length of both adrenal glands. Although the ICC of our study had poor-to-fair interrater reliability in measurement of thickness and width of both adrenal glands, we found that the difference of measurement in adrenal gland size between the two readers was < 1 mm, suggesting no real essential impact on clinical practice. From our results, we recommend a 95th percentile cutoff value from both readers' data to determine the maximum normal size of the adrenal gland as shown in Table 1. Montagne JP et al.[7] suggested that adrenal limb width was the most useful of the measurements in clinical practice. The 95th percentile of normal size adrenal limb width in our study were as follows: 1) right adrenal gland: axial width medial limb 4.6 mm., coronal width medial limb 4.8 mm., axial width lateral limb 4.6 mm., coronal width lateral limb 4.3 mm. and 2) left adrenal gland: axial width medial limb 5.7 mm., coronal width medial limb 5.7 mm., axial width lateral limb 4.8 mm., coronal width lateral limb

4.6 mm. Interestingly, the measurement of thickness and limb widths of the normal adrenal gland in the coronal plane was first described in our study. Thus, either the axial or coronal plane can be used for determining the size of the adrenal glands, depending on which plane shows better delineation.

For frequency of the location, the right gland was slightly more frequently cephalad to the left gland, similar to the previous study by Montagne JP et al.[7]

Our study illustrated that the inverted Y shape was the most common shape of both right and left adrenal glands, 85.8% and 93.7%, respectively. The less common shapes were inverted V and linear shapes. Our result was the same as Karstaedt N et al.,[8] that found most common type was inverted Y. However, our study did not find a triangular shape among normal adrenal glands.

Finally, our study revealed that all normal adrenal glands had homogeneous enhancement in the arterial and nephrographic phases.

There were some limitations in our study. First, we could not perform CT of the abdomen on normal volunteers because of the radiation exposure and the associated ethical issues. Our study chose the population of potential renal donors that were available for CT of the abdomen and most resembled the normal population. Second, the cut-off value that we used, the 95th percentile of normal-sized adrenal gland, was not evaluated in its accuracy in distinguishing between normal and pathologic adrenal glands in clinical practice. Further investigation is required to determine the accuracy of this criterion.

CONCLUSION

The majority of normal adrenal glands were right relatively cephalad to left, inverted Y shape, and homogenous enhancement. The 95th percentile of size normal adrenal gland should be used as a cutoff value to differentiate between normal and thickened limbs and body.

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The Effect of Group Cognitive Behavioral Play Therapy Program on Self-Control among Late Childhood in Foster Care

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ABSTRACT

Objective: To develop and evaluate the effectiveness of group cognitive behavioral play therapy program on self-control in foster care for late childhood.

Methods: The program was developed by researchers and validated by 3 experts, and consisted of one individual session and 14 group sessions. Twelve boys with age 8-10 years at Mahamek Home for Boys attended the 40-50 minutes group session twice a week for 7 consecutive weeks. The effectiveness of the program was evaluated pre and post- intervention by using Self-Control Skill in 5 Situational Test and externalizing domains in Thai Youth Checklist (TYC). Descriptive statistics, Wilcoxon Signed Ranks Test, and qualitative analysis were used in data analysis.

Results: The participants were 12 boys with age 8-10 years at Mahamek Home for Boys. They were studying primary education in public school and were fostered because of poverty, abandonment, and abuse. Comparison of changes between pre and post-intervention scores revealed increment in mean score of Self-Control Skill in 5 Situational Tests with p-value at 0.267, related to decrement in mean score of overall externalizing behavior problems in TYC with p-value at 0.086. Examined score in sub-domains of TYC found statistical decrease in aggression with p-value at 0.028 (p. <.05) as well as decrease in delinquency and impulsivity with p-value at 0.257 and 0.146, but found slightly increased immaturity domain with p-value at 0.892.

Conclusion: The program was effective in reducing aggressive behavior and had a tendency to improve self-control skill among 8-10 years old children in a foster care. However, the effectiveness of the program in long-term needs to be further investigated.

Keywords: Self-control; group cognitive behavioral play therapy; late-childhood; foster child (Siriraj Med J 2018;70: 507-513)

INTRODUCTION

Self-control is an ability to regulate emotions, thought and able to inhibit inappropriate behavior with any situation. It relates with academic achievement, social skill and can predict future success. The one with less self-control skill will be easily distracted, impulsive, unable to wait and have difficulty to deal with frustration. This leads to poor academic performance and social problems such as violence, delinquency or drugs addiction in adolescent.[1,2]

Self-control is cumulative behavior which can be promoted by learning process. The first place to learn is family. Children learn to regulate and manage emotion from parents by observing parent's behavior, imitating and maintaining it from reinforcement.[3,4] Prior studies found that a child who came from lack of warmth and love

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family tends to lack self-control skill and has tendencies to become delinquent child.[3] According to Piaget, who mentioned that development of self-control behavior should be developed by their own experience? The more behavior feedback he gets, the more chance he has learned. Besides cumulative behavior, self-control involves with cognitive development. They must be able to understand the consequence of action and outcome and be able to remember what behavior leads to reward or punishment and make decision by their own experiences.

Cognitive behavior play therapy or CBPT was developed by Knell in 1998. She applied play technique with cognitive behavior therapy by using toys and play as a medium to child's thought and reflect child's behavior. In this technique, a child learns the connection of emotions, thought and behavior, by applying learn CBT techniques such as labelling feelings, coping skill via play. Learning through play is fun, understandable and concrete. Moreover, playing with others is an opportunity for a child to learn about the consequence of his own action from other members' response or from a therapist who provides feedbacks and modelling. After a child receives direct or indirect feedbacks which are not matched with his prior belief, he tends to re-evaluate his action and alter behavior to be more appropriate.[6-8] However, conventional leaning in primary school age children may have limitation due to cognitive development level

All the mentioned above points that if children who are at risk for externalizing behavior problems, have a chance to learn new appropriate behavior through CBPT their self-control skill may be improved. However most of prior interventions were conducted in normal population. We still need to explore the appropriateness of using this type of intervention in the specific populations, which have many different characteristics like severity and complexity of mental health problems.[9] Therefore, the objective of this study was to develop and evaluate the effectiveness of group cognitive behavior play therapy program with expectation of increment in self-control skill and decrement in externalizing behavior problems among foster children.

MATERIALS AND METHODS

This study was designed as quasi-experimental research, one group pre-test-post-test design for evaluating the effectiveness of the program on self-control among late childhood in foster care. This study was conducted with the approval of the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University (Si 606/2017) with the following procedure.

Participant

Participants in this study were 12 boys with age from 8-10 years old at Mahamek Home for boys. This number was according to Marvin E. Shaw (1971) who explained that a group should have at least 10 members but not exceed 13 people, for thorough participation in the activity.[10] Mahamek Home for boys is a foster home for 6-18 years old boys with poverty, abandonment, or abuse. This home provide shelter, food, basic education in public school, medical care, and recreation activities. Thus, it is suitable to examine the effectiveness of the psychological intervention program.

Inclusion criteria

1. Age from 8-10 years old through studying period
2. Had higher score in externalizing domain of Thai Youth Checklist than the population in Mahamek Home for Boys' average score
3. Was not currently receiving other intervention.
4. Willing to join the study.

Exclusion criteria

1. Subject has intellectual disability, which was accessed from their profile.
2. Subject has other serious mental or physical disorder which would affect learning from the program.

Withdrawal criteria (Subjects were allowed to withdraw from the study in any period)

1. Participated in program less than 80%.

Instruments

Instruments which were used in this study were divided into 2 parts which were intervention instrument and data collection instruments.

Intervention instrument

Group cognitive behavioral play therapy program

The program was developed by researchers and validated by 3 experts based on theory and techniques from cognitive behavior play therapy, group play therapy, and self-control development (program activities were displayed in Table 1). The program emphasised on learning and practicing in emotional and thought awareness, problem-solving skill and reinforcement of desired behavior by using token economy technique. Feedback in each session and homework assignment were used to evaluate participants understanding of the program.

This program consisted of 1 individual session and 14 group sessions, conducted in 40-50 minutes, twice a week. The program was organized in 3 phases.

TABLE 1. The program activities.

Phase/ session		Goal	Activity
Initial	0	Enhancing relationship and explanation of activity	Drawing, playing individual game and making time chain in individual session
	1	Enhancing relationship and emotional observation	Clay work and emotional face
	2	Learning the consequence of emotional and physiological changing	Roleplay with emotional guessing game
	3	Learning the consequence of thought and emotional	Situational and thought bubble drawing
	4	Applying CBT model to self-observation and exploring consequence of behavior	Balloon blowing till it burst
	5	Applying CBT model to self-observation and use internal cues as a sign to control own behavior	Balloon blowing till it big but not burst
Middle	6	Teaching cognitive reappraisal technique and giving an opportunity to dealing with emotional arousal	Control land: Do not smile and laugh game
	7	Teaching problem-solving technique	Catch the dragon's head and tail game
	8	Learning to stop before action by using technique for clam down emotional and use problem-solving technique	Janga tower game with "stop-think-act" technique
	9	Applying technique in frustration situation I	Traffic treasure game: follow the traffic light while crossing intersection to collect the treasure
	10	Applying technique in frustration situation II	Tower building while facing with distraction situation
	11	Applying technique in frustration situation III	Bingo game while facing with waiting and frustration situation
	12	Applying technique in frustration situation IV	Roleplay
Terminal	13	Reviewing and conclusion	Board game
	14	Feedback, self-change observation and terminate	Feedback activity, giving reward and goodbye letter

1. The initial phase aimed to enhance a relationship between group members, provide basic concepts about CBT model, and help the participants to understand how to observe their own emotions, thought and behavior through play activities and guidance. This phase consisted of one individual session and group sessions from the first to fifth.

2. The middle phase consisted of 2 sub-phases. The objective was to teach self-control techniques and facilitate participants to use these techniques in play activities. The second objective was to practice techniques and receive feedbacks form therapist and other members. This phase consisted of the activities in sixth to eleventh sessions.

3. Terminal phase was aimed to summarize, provide feedback about program and discuss the changes of themselves and other members. This phase also provided time for farewell and termination of the program. It consisted of the 13th-14th sessions.

Data collecting instruments and analysis

Part 1: Demographic data consisted of age and classroom, and collecting data from participant's caretaker.

Descriptive statistics was used to analyze demographic data in mean and percentage.

Part 2: Self-control behavior consisted of Self-Control Skill in 5 Situations Test and Thai Youth Checklist. Data were collected in pre and post intervention 7 weeks apart.

Self-Control Skill in 5 Situational Test was developed by the authors and content validated by 3 experts. It was based on concepts and theories of self-control, and cognitive development. This test consisted of 5 open ended questions asking about tendencies of behavior that may manifest if participants are in these 5 situations. For instance, *if you plan to have lunch at your favorite store, but it has a very long queue, what will you do?* Score 1 if the answer reflects to responsibility, perform behavior that along with moral and social norm, able to inhibit inappropriate behavior, refuse temptation which may lead to negative impact. Score 0 if the answer is opposite from above. Data were collected from participants. For participants who could not read, they were facilitated by research assistants. Wilcoxon Signed Ranks Test and qualitative analysis were used in data analysis.

Thai Youth checklist (TYC) is a screening for observational behavioral problems in children age 6-17 years old, and classify behavior problems in 2 domains, internalizing and externalizing problems. TYC has 2 forms; observing behavior by parents at home and teachers at school, both must be the one who has high interaction with a child for direct observation. It was translated to Thai by Suwannalert S et al; 1989, and test-retest and inter-interviewer reliability were 0.81 and 0.91.[11] The externalizing behavioral problems in boys age 6-11 years old: parent version was used in this study. This domain consists of four sub-domains; aggressive, impulsivity, delinquency, and immaturity. Data were collected from 4 caretakers who have 8-12 boys in care. Each caretaker was assigned to observe and report behavior of 2-5 children according to the number of participations in care. The data was analyzed by using Wilcoxon Signed Ranks Test.

RESULTS

Demographic data

Participants were 12 boys, age 8-10 years old in Mahamek Home for Boys, who had scored from TYC higher than the average score of this population. Two participants were excluded because they participated less than 80 % and 1 participant was excluded because the researcher was unable to collected data of post-intervention. Therefore, the number of participants in this study was 9. The participants were 4 boys with 8 years old, 4 boys with 9 years old and 1 with 10 years old. All participants study in public school, one boy in grade one, five in grade two and three in grade three. They came to foster care because of poverty, abandonment and had been abused. General information of the participants was displayed in Table 2.

TABLE 2. General information of the participants.

ID	Age	Shelter	Number of participations	Noted
1	10	A	9/14	Excluded
2	10	A	9/14	Excluded
3	9	B	13/14	
4	9	B	12/14	
5	10	B	12/14	
6	8	B	12/14	
7	8	C	12/14	Excluded
8	8	C	14/14	
9	8	C	13/14	
10	8	D	13/14	
11	9	D	13/14	
12	9	B	11/14	

The effect of group cognitive behavior play therapy on self-control

Self-control skill in 5 situational tests The result from Table 3 showed increasing of mean score in post-intervention, with non-statistical significance (Z = - 1.134; p-value = 0.257) However, there were some interesting changes in participants' answers for instance; *if your friend dislikes your drawing in group work and erases it, what will you do?* The answer in pre-intervention such as *"punch him" "damn him"* changed to *"tell a teacher" "tell a friend to stop erasing and draw again"* Nonetheless, there were answers which implied lack of self-control skill like, *"Ask a friend who is in the queue to buy for me" "pay money for jump the queue"* for the situation like *if you plan to have lunch at your favorite store, but it has a very long queue, what will you do?*

Thai Youth Checklist Comparison of changes between pre and post-intervention found the mean score of overall externalizing behavior problems was decreased with non-statistically significant level (Z = - 1.719; p = 0.086). Examination of the mean score of subdomains found decreases in aggression, impulsivity, and delinquency (Z= -2.203; p = 0 .028 / Z= -1,452; p = 0.146 / Z = -1.132; p = 0.257), but only aggression was statistically changed. However, the score of immaturity subdomain was increased. (Z = .136; p = 0.892), as displayed in Table 4.

DISCUSSION

The results show that this program is effective in decreasing aggressive behavior (p. <.05). It also has a trend to improve self-control skill and reduce externalizing behavior problems. The positive result, especially in aggressive domain, is congruent with previous studies which found that group cognitive behavior therapy is effective in reducing aggressive behavior and externalizing behavior in primary and secondary school students[5,12] and in street girls.[13] All these congruent results may conclude that group cognitive behavior therapy is also effective treatment in enhancing self-control skill and reducing externalizing behavior problems in special populations like this one.

There are studies which found that perception bias of anger and sadness, ability in coping with anger, and inappropriate expression of sadness relate with externalizing behavior problems[14-16]. It is the same with the participants' behavior. We found that they got angry easily even from slight mistake or by accident and always immediately reacted with aggressive behavior such as, destroying things or get into fight. It is interesting that they did the same behavior when they might be unsatisfied, for instance, not been chosen to do extra activity. However, when they were asked about their feeling, none of them mentioned sadness, most of them reported anger. Therefore, decreasing of externalizing

TABLE 3. Comparing the mean score of self-control skill in 5 situations test.

Time	Mean	S.D.	Z[a]	P-value
Pre	4.11	1.364	-1.134	.257
Post	4.67	.707		

TABLE 4. Comparing of mean score of TYC.

Domain	Time	Mean	S.D.	Z[a]	P-value
Immaturity	Pre	4.56	2.40	.136	.892
	Post	4. 67	2.39		
Delinquency	Pre	4.33	2.55	-1.132	.257
	Post	3.33	2.73		
Impulsive	Pre	7.11	3.37	-1.452	.146
	Post	6.22	2.05		
Aggressive	Pre	17.22	4.73	-2.203	.028*
	Post	11.89	6.15		
Total	Pre	29.78	9.65	-1.719	.086
	Post	24.33	10.95		

Abbreviations: TYC=Thai Youth Checklist

behavior problems might be because their emotional awareness and coping skill were improved. As shown in the answer of participants from activities, worksheet, and interviewing for instance; *"I used to be a bad temper person, but now I learned how to manage it, especially when I get in fight with my friends", "I always say a bad word to people around me when I felt angry in the past but I seldom do it now. Instead of saying bad word I count 1-10, think of other thing to calm myself down"*.

Only significant improvement in aggression domain may be due to participants' behavior. We found that they felt angry more often than other emotion and always manifested with aggressive behavior in group sessions. Therefore, participants had more chance to learn the consequences of aggressive behavior by direct or indirect reinforcement from the program. This could lead to cognitive and behavior adaptation by accommodation process which would occur when new experiences conflicts with existing belief.[17]

Examination of the answer of the participants from Self-Control Skill in 5 Situations Test revealed that most of the answers from the post intervention changed to become more appropriate. This reflected that changes in cognition occurred. However, behavioral change needs more time to practice and acquire experience in various situations. Therefore, practicing only in 14 group sessions without assistance from caretaker to generate learned skill or techniques in daily life situations might not be enough for behavior changing.

Age could be another factor which affected the result. From the behavior observation and worksheet, we found that participants with age 10 years old perform better in cognitive reappraisal and problem-solving technique than those younger. This could be effect of children's cognitive development, which develops by age. This finding is congruent with the study of DeCicco, et al (2014) who found that effectiveness of cognitive reappraisal techniques is increased by age[18] and the prior meta-analysis supports that CBT has more effectiveness when applied with older children.[19]

However, it does not mean that younger participants are not able to learn from these two techniques. Prior studies found that 8-year-olds could understand the connection of cognitive reappraisal and emotion adaptation.[20-21] As with this study, we found younger participants understood and applied techniques better if they received intimate advice, modelling or more thinking time. Therefore, if they get more guidance or the duration of the program was extended, their behavior may be improved.

A relaxation technique, was found to be effective in reducing aggressive behavior among elementary students with emotional or behavioral disorders.[22] In this study we found most of the participants understood, did well in practicing, but had some limitations in applying to some frustrated situations. This could be the effect of inadequate practicing, because this technique may not be able to apply in daily life effectively unless they have already become familiar with it. Hence, extending the duration of the program may increase the program's effectiveness.

Another factor that may enhance the program effectiveness is including caretaker in the program. There are evidences from studies which found that including parents in a program can enhance effectiveness in reducing externalizing behavior problems of children. Parents can facilitate children to use learned techniques in daily life and reinforce trained behavior which supports, generalises and maintains desired behaviors.[23-24] There are studies which found that relative foster care is superior to non-relative foster care, because of pre-existing emotional and social connection and quality of interaction with foster parents can predict externalizing behavior problems.[24] However, including foster parents in the program may have limitation in practice because foster parents may have limited time to participate in the program.[24] Therefore, incorporating foster parents into the program should be investigated more in further study.

Considering about observers may be the factor which lead to different outcome. According to previous meta-analysis study which found that parents tend to report larger improvement in externalizing symptom than teacher. This because parents and teachers may differ in level of intimacy and concern.[5] For this study, the responder was foster parents who have 8-12 children under care. This number may be too large for close interaction with each participant. This may affect to behavior observation, particularly in minor detail such as crying or blathering. However, aggression is easy to notice or receive complaint, which might not be affected from this limitation.

The questionnaire may be another factor which affected to the statistical change in aggressive domain. Items of Thai Youth Checklist are unequal in each domain, the highest item domain is aggression. It is possible that responders might have more chance to notice and report the changing of aggressive behavior than others. Thus, aggression is the only domain that was statistically significantly decreased. For the self-control skill test, which has only 5 items and participants scored high in pre-intervention period ($\bar{X}$= 4.11), it is limited in

quantitative evaluation. From this consequence, it is possible that different outcomes might be due to the limitations of the assessment.

Finally, generalization of this study is limited. This study was investigated with 8-10 years boys in foster care in one group design without booster session. Thus, we could not compare the program effectiveness with external factors or other populations as well as the long-time effectiveness which still needs to be further investigated.

CONCLUSION

The program was effective in reducing aggressive behavior and had a trend to improve self-control skill among 8-10 year old children in foster care. However, this program needs to be developed. Including foster parents into the program, review new assessment, and investigation in controlled experiment with booster session design are recommended for further research.

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The Teratogenic Effects of Monosodium Glutamate (MSG) on the Development of Chick Embryos

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ABSTRACT

Objective: The present study was conducted to evaluate the toxicity and teratogenic effects of MSG in chick embryo development.

Methods: Various concentrations of MSG solution, 1 mg, 1.5 mg, 2.5 mg and 3 mg of MSG/gm egg weight were injected into the fertilized hen's egg at 21 hrs of incubation and further incubated until day 3, 6 and 10. The day 3 and day 6 were processed for serial section, while the day 3 was processed for total mount and day 10 were observed totally.

Results: The effects of MSG were growth retardation, embryonic death and congenital malformations of several organs such as brain (rachischisis and anencephaly), eye (microphthalmia and anophthalmia), ectopiacordis, ectopia viscera, opening of posterior neuropore (spinal bifida) and under development of heart and viscerae.

Conclusion: MSG produced congenital deformities and growth retardation in chick embryos which may predict to cause the same results to human embryos. Pregnant women should be advised not to consume MSG to avoid potential harmful effects to the unborn child.

Keywords: Monosodium glutamate (MSG); teratogen; chick embryo (Siriraj Med J 2018;70: 514-522)

INTRODUCTION

MSG is the most commonly used ingredient for cooking process in many countries. In United Kingdom, people consumed MSG about 4 grams (less than one teaspoon) per week while the U.S. showed 0.55 grams per week, while Taiwan reported a higher level of about 3 grams per day.[1,2] MSG is a natural neurotransmitter of the brain and induced symptoms known as "Chinese restaurant syndrome" which can be detected after the consumption of Chinese meals. It is characterized by headache, chest discomfort and facial flushing due to abnormal function of glutamate receptors. MSG was reported to relate with neurological diseases especially retinal degeneration, brain trauma and endocrine disorder causing obesity and gonad dysfunction.[3,4] The excessive dose and long term usage of MSG in cooking affect tissue organ and embryonic development if it is taken by pregnant women during pregnancy.[5] To confirm the teratogenic effects of MSG, it was easier to use chick embryo because there were plenty of databases compared with human (Hamburger and Hamilton, 1952).[6] Chick embryo is available all year round with short gestation time and also inexpensive, so chick embryo is an excellent animal model in developmental field.

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In 2012, Fatma and Anan studied the effects of MSG on chick embryo and reported different congenital malformations such as growth retardation, subcutaneous bleeding, abdominal hernia, brain deformation, monophthalmia and beak malformation. Histological study of liver in the treated group showed less cell density and a dilation of venous canals and blood sinusoids, fibrosis, bleeding, hemorrhage and congestion, lipid droplets and necrosis. It was concluded that MSG caused growth retardation, congenital malformation and liver degeneration,[1] although they showed no illustration of such malformations except for the histology of liver. In 2016, Mahaliyana *et.al.* studied toxicity of MSG on embryonic development of zebrafish (*Daniorerio*).There was no observable malformation in low dose group. However, at higher concentrations there were abnormal developments such as growth retardation, shrinkage of the chorion, yolk sac edema, lack of pigmentation, tail deformities and scoliosis.[7] As in the previous teratogenic studies the toxicity and teratogenic effects of MSG were still unclear, the present study was conducted to study the toxicity and teratogenic effects of MSG on the 3rd, 6th and 10th day chick embryos which were injected by different concentrations of MSG into the yolk sac of fertilized egg. The mortality rates were recorded and the teratogenic results were revealed by gross structure anomalies and histological changes of chick embryos by total mount and serial sections of the experimental groups compared to the control group.

MATERIALS AND METHODS

Ethics Statement

According to German animal care guidelines, no IACUC approval was necessary to perform the embryo experiments. According to the local guidelines, only experiments with chick embryos E18 and older need IACUC approval. However, the embryos used in this study were all in early stages of embryonic development (between E1 and E11).[8]

ACUC Guideline

The Use and Euthanasia Procedures of Chicken/ Avian Embryos Avian embryos are not considered live animals under PHS policy. However, there is a consensus in the scientific community that at a certain point in development, avian embryos can experience pain. Because that exact point is not known for chicken embryos, chicken use and euthanasia guidelines differ across institutions. Cal Poly Pomona has chosen to adopt a guideline with the belief that pain occurs on or after gestation day 13, in anticipation of reviewing protocols including them. However, this study used earlier stages of chick embryos, day 3-day 10.[9]

Fertilized eggs of white leg horn hen (*Gallus gallusdomesticus*) were collected fresh from the Department of Animal Science, Faculty of Agriculture, Kasetsart University. They were divided into 2 groups, the control group (n = 6) was injected with 0.15 ml of normal saline and treated groups (n = 60) were injected with 0.15 ml of the MSG solution with the concentrations 1 mg, 1.5 mg, 2.5 mg and 3 mg of MSG/gm egg weight into the yolk sac before incubation. The eggs were cleaned with 70% ethanol and incubated at 37°c. After 21 hours of incubation, all eggs were removed from the incubator and cleaned at the blunt ends of the eggshell with 70% ethanol before injecting into the yolk sac with the equal volume of 0.15 ml of NSS and the MSG solutions. After injection, the eggs were further incubated until day 3, 6 and 10. Embryos were removed from 2 eggs of control group and 5 eggs of each treated group for observing embryonic abnormalities and mortality. The day 3 embryos were processed for total mount and serial section while day 6 were processed for the serial section and day 6 and 10 embryos were processed for removing of visceral organs and alcian blue and alizalin red staining of cartilages and bones. Then the surviving chick embryos were observed for their external morphology indicating any gross malformations and more detail of malformations were confirmed by the serial section.

RESULTS

The mortality rate of the day 3 chick embryos

The living embryo was indicated by observing the heartbeat and blood circulation. The percentages of survival and mortality were presented in Table 1.

The results showed the lowest mortality rate was 0% in the control group and the MSG 1 mg/gm egg weight groups. The mortality rates were increasing from 20% to 40% in the MSG concentration of 1.5 to 2.5 and 3 mg/gm egg weight, respectively. The dead embryos did not exceed 50% in all groups.

The mortality rate of the day 6 chick embryos

The living day 6 chick embryos were indicated by observing the heartbeat and blood circulation. The percentages of survival and mortality were presented in Table 2.

The lowest mortality rate was 0% in the control and the MSG 1mg/gm egg weight groups, 20% in MSG 1.5 mg/gm egg weight group and 40% in 2.5 and 3 mg/gm egg weight groups, respectively. The dead embryos did not exceed 50% in all groups.

TABLE 1. Percentages of the survival and mortality rates of the day3 chick embryos of the experimental groups compared with control group

Group (mg/gm egg weight)	Survival (%)	Mortality (%)
Control (n=5)	100 (5)	0 (0)
1 (n=5)	100 (5)	0 (0)
1.5 (n=5)	80 (4)	20 (1)
2.5 (n=5)	60 (3)	40 (2)
3 (n=5)	60 (3)	40 (2)

TABLE 2. Percentages of the survival and mortality of the day 6 chick embryos of the experimental groups compared with the control group

Group (mg/gm egg weight)	Survival (%)	Mortality (%)
Control (n=5)	100 (5)	0 (0)
1 (n=5)	100 (5)	0 (0)
1.5 (n=5)	80 (4)	20 (1)
2.5 (n=5)	60 (3)	40 (2)
3 (n=5)	60 (3)	40 (2)

The mortality rate of the day 10 chick embryos

The percentages of survival and mortality of the day 10 chick embryos were presented in Table 3.

The lowest mortality rate was 0% in the control, 20% in 1 and 1.5 mg/gm egg weight groups and 40% in 2.5 and 3 mg/gm egg weight groups, respectively. The dead embryos were not more than 50% in all groups.

Then the surviving chick embryos were observed for the external morphology indicating any gross malformations and more detail malformations were confirmed by serial section of the suspected organs. The cartilage and bone malformations were indicated by the alcian blue /alizarin red staining of the eviscerated embryos.

TABLE 3. Percentages of the survival and mortality of the day 10 chick embryos of the experimental groups compared with control group

Group (mg/gm egg weight)	Survival (%)	Mortality (%)
Control (n=5)	100 (5)	0 (0)
1 (n=5)	80 (4)	20 (1)
1.5 (n=5)	80 (4)	20 (1)
2.5 (n=5)	60 (3)	40 (2)
3 (n=5)	80 (4)	20 (1)

The total mount of the day 3 chick embryos (Fig 1)

The total mount of the day 3 chick embryos showed that the control group had normal development as classified by Hamburger and Hamilton[10] as stage 18 (36 somites) which the brain formation composed of the 5 brain vesicles (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon). There were 2 flexures (cervical flexure and cephalic flexure). The body of embryo was rotated to the right side. Normal optic cup at diencephalon was large, horse-shoe shaped and lens located at the middle of the optic cup. The heart was S-shaped. There were 4 branchial arches. Anterior and posterior limb buds were presented. Somites extended to the caudal end and the neural tube appeared as two parallel dense lines.

The experimental group of 1 mg of MSG/gm egg weight (B) showed retardation of development of the brain with the development of only one flexure (cephalic flexure). The optic cup and lens were smaller than the control. The branchial arch showed only one. The heart was irregular U-shaped with dilated lumen. There was irregular twisting of the body. The somites did not extend to the caudal end and the neural tube appeared as non-parallel dense lines. The limb buds and tail fold were absent. The (C) 2.5 mg of MSG/gm egg weight. (AN = Anterior neuropore, H = Heart) and 3 mg (D) of MSG/gm egg weight showed common malformations, the opening of anterior neuropore, the absence of eye primordia (anophthalmia), the absence of branchial apparatus, the dilated heart loop, retardation of somite formation and the absence of limb buds and tail fold.

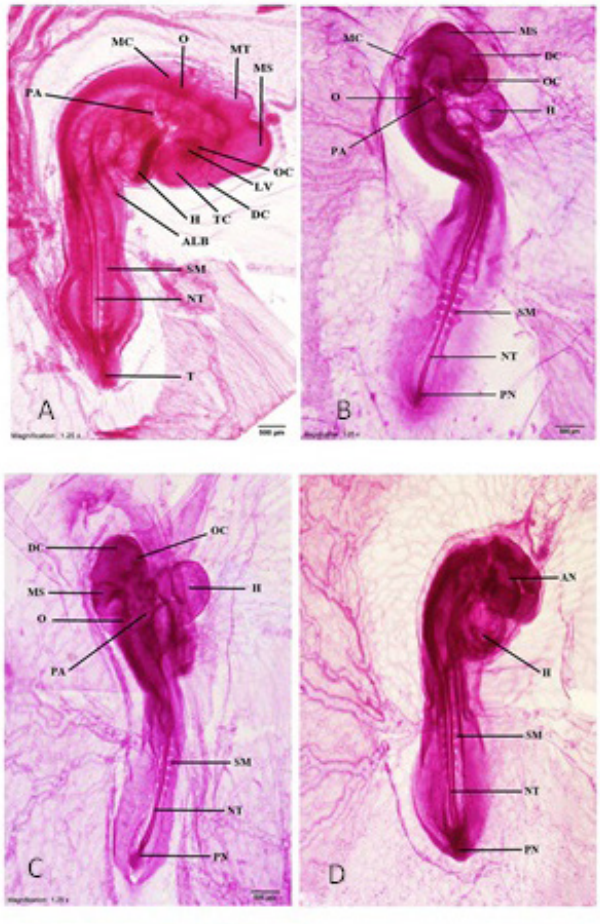


Fig 1. Total mount of day 3 chick embryo of the control group (A), (B) the experimental group of 1 mg of MSG/gm egg weight. (C) 2.5 mg of MSG/gm egg weight. (D) 3 mg of MSG/gm egg weight. **Abbreviations**: TC=Telencephalon, DC=Diencephalon, MS=Mesencephalon, MT=Metencephalon, MC=Myelencephalon, OC=Optic cup, LV=Lens vesicle, O=Otocyst, H=Heart, PA=Pharyngeal arch, ALB=Anterior limb buds, NT=Neural tube, SM=Somite, T=Tail fold, PN=Posterior neuropore, AN=Anterior neuropore.

The eye of the day 3 (Fig 2)

The eyes of the control comprised optic cup, optic stalk and lens vesicle. The optic cup was large and oval shaped structure connected with the diencephalon by the optic stalk. It was composed of the outer pigment layer and inner nervous layer which lied in closed contact to obliterate the intraretinal space. The lens was composed of cuboidal cell layer, the anterior lens epithelium, and the elongated cell layer, posterior lens fiber. All experiment groups showed more or less growth retardation represented by smaller optic cup with wide opening of the intraretinal space, with separation between the outer pigment and inner nervous layers. The lenses were smaller with the persistence of lens pit in the 1.5 and 2.5 mg groups (Fig 2C and 2D). All experiment groups showed no elongation of the posterior lens fiber.

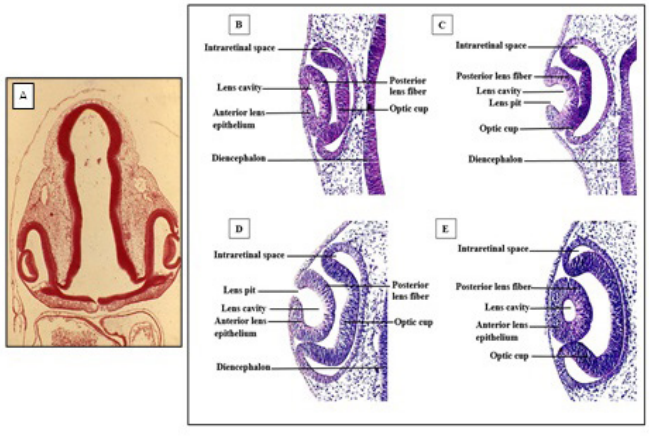


Fig 2. The micrographs of the serial section of the day 3 chick embryos at the level of the eyes showed the control group in Fig A and the experimental groups in Fig B and E were dose 1 mg and 3 mg of MSG/gm egg weight showed wide separation of intraretinal space, Fig C and D were dose 1.5 mg and 2.5 mg of MSG/gm egg weight showed opening of the lens pit or incompleted fusion of the lens placode.

The external morphology of day 6 chick embryos (Fig 3)

The external morphology of day 6 chick embryos (HH stage 29) revealed the diameter of head was larger than the body. Eyes were large round-shaped and symmetrical. Beak appeared prominent but absence of egg's tooth. The heart and viscerae situated in the thoracic and abdominal cavities was covered by the skin. Upper and lower limbs were paddle-shaped with no sign of the formation of digits.[10,11,12]

The results showed normal external morphology in the control group while experimental groups showed congenital malformations and growth retardation in all experimental groups. The abnormalities comprised opening of anterior neuropore (anencephaly), absence of eye (anophthalmia), small eye (microphthalmia). The visceral organs situated outside the body cavity (ectopia viscera). The heart situated outside the thoracic cavity (ectopiacordis) and opening of posterior neuropore.

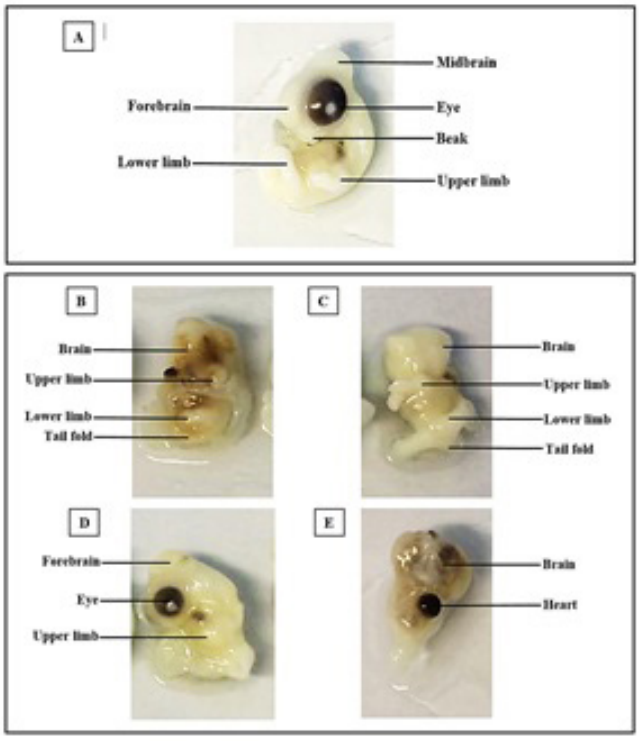


Fig 3. The photographs of the day 6 chick embryos showed normal development of the control group in Fig A and showed congenital malformations in all of the experimental groups in Fig B was 1 mg treated group, showed opening of anterior neuropore called anencephaly, small eyes (microphthalmoa) and ectopia viscera, Fig C was 1.5 mg, showed absent both eyes called anopthalmia and opening of the anterior neuropores, Fig D was 2 mg, showed opening of both anterior and posterior neuropores and Fig E was 3 mg, showed opening of anterior neuropore called anencephaly and ectopia cordis.

The eyes of day 6 embryos indicated in the serial section (Fig 4)

The eye of the control group showed normal development indicated by Fig A. The optic cup was oval-shaped and comprised outer pigment layer and inner nervous layer which lied close to each other. The intraretinal space was obliterated. The lens comprised anterior lens epithelium which appeared as simple cuboidal epithelium and posterior lens fiber which elongated dramatically and obliterated the lens cavity.

All experimental groups showed more or less abnormalities of the eye development. Some embryos of the experimental group of dose 2.5 mg of MSG/gm egg weight (Fig 4C) showed retardation of eye development indicated by the persistence of the intraretinal space

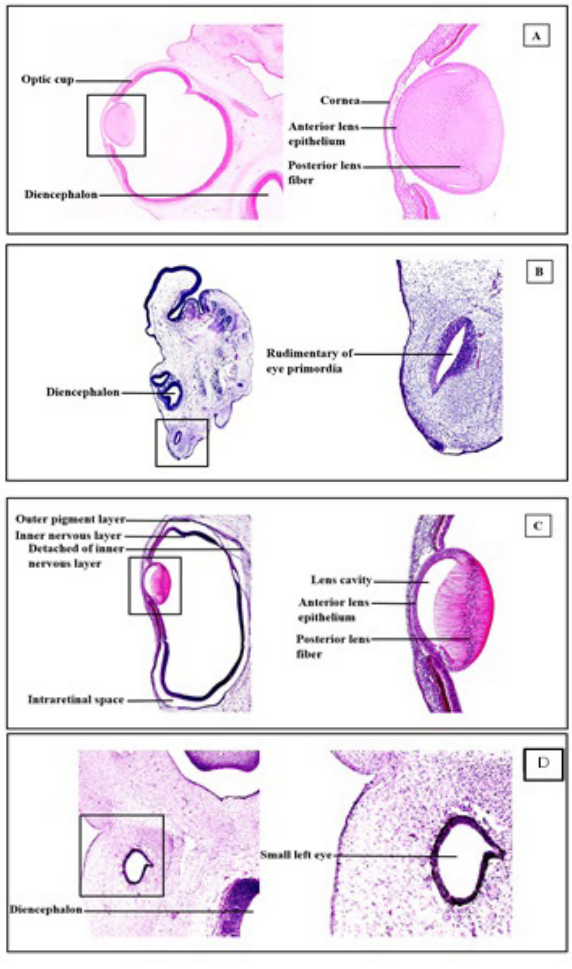


Fig 4. The micrographs of the development of the control (4A) and the experimental groups of dose 1 mg of MSG/gm egg weight in Fig 4B, showed under development of diencephalon and absent of both eyes (anophthalmia), (4C) the experimental group of dose 2.5 mg of MSG/gm egg weight showed retardation of eye development indicated by the persistent of the intraretinal space and the lens cavity and Fig 4D was the group 3 mg of MSG/gm egg weight showed abnormal development indicated by the absent formation of both eyes (anophthalmia) associated with the abnormality of diencephalon.

and the lens cavity. Fig 4B and Fig 4D were the 1 and 3 mg of MSG/gm egg weight which showed abnormal development of brains together with absent formation of both eyes (anophthalmia).

The abnormal development of mesonephros and gonad (Fig 5)

The control group indicated in Fig 5A, showed a large pair of mesonephros comprised of numerous mesonephric tubules and one duct with glomeruli situated at the medial aspect. The gonad was large mass attached to the medial surface of the mesonephros. All experiment groups showed abnormality of mesonephros and gonadal ridge as indicated in Fig 5 B-D and showed very small mesonephros with fewer tubules and also very small gonadal ridge. Fig 5D showed large cystic cavities and Fig 5E showed rudimentary of mesonephric tubules with cystic formation.

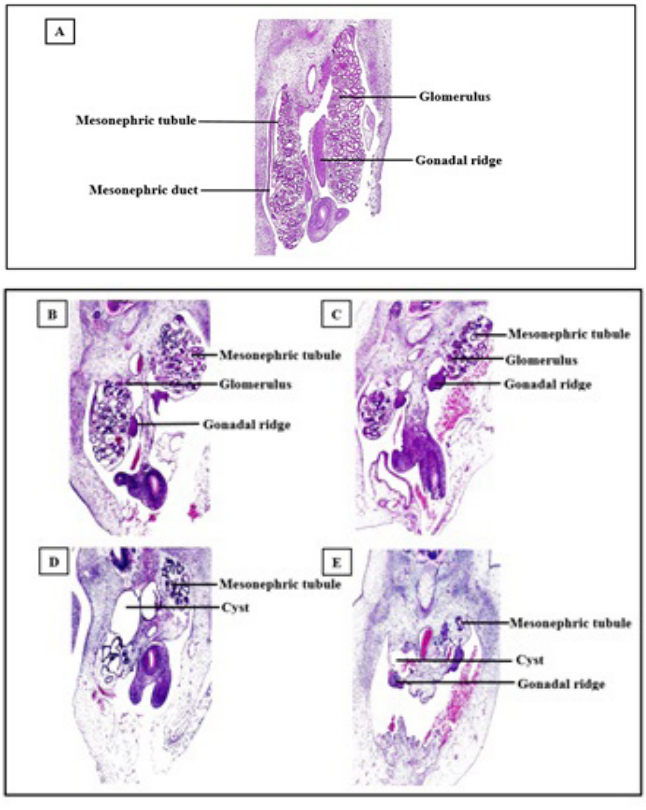


Fig 5. The micrographs of the development of mesonephros and gonad of the control group in Fig A and the experimental groups (B, C, D and E) showed more or less abnormals development, Fig B and C (1, 1.5 mg) showed small size and underdeveloped of mesonephric tubules and corpuscles with small gonads, Fig D and E (2.5 and 3 mg) showed more abnormality, apart from the small size and lesser number of tubules and absent of mesonephric corpuscle, there were large cystic formation and very small gonad.

Common abnormalities among all treated groups
The serial section of day 6 embryo

1. Dosage of 1 mg of MSG/gm egg weight (Fig 6) group showed widely opened of anterior neuropore and unidentified portion of the forebrain (Fig 6 A), unidentified eye at the level of diencephalon (anophthalmia) and absent otocyst (Fig 6B), and unfusioned spinal cord. At the level of thoracic and abdominal cavity it showed small stomach and dilated liver cord (Fig 6C). The ectopia cordis (Fig 6C) had underdeveloped heart. There was thinned wall atrium and ventricle with unfused endocardial cushion (Fig 6C). At the level of mesonephros and gonad it showed under development of the organs with irregular smaller mesonephric tubules without gonad (Fig 6D).

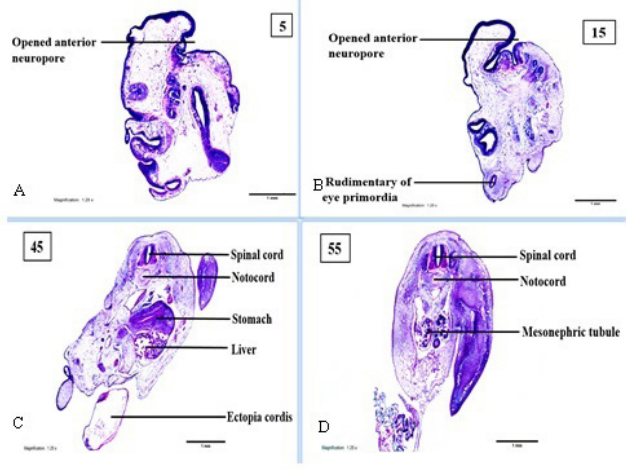


Fig 6. A, B, C, D were serial sections of day 6 chick embryo injected with 1 mg of MSG, section number 5, 15, 45 and 55, respectively to show multiple malformations in the same embryo. Fig 6A and 6B were at the head region to show irregular shaped brain, 6B showed the side opening of anterior neuropore. Fig 6B showed irregularity of diencephalon with rudimentary eye primordium beside. Fig 6C showed small viscerae within the body cavity and the heart situated outside (ectopiacordis). Fig 6D showed small and under developed mesonephros. This embryo showed anencephaly, anophthalmia, ectopiacordis and under developed of all visceral organs.

2. Dose 1.5 mg of MSG/gm egg weight (Fig 7)

The serial section showed irregular and unidentified part of brain, nasal cavity at the ventral portion, opening of brain at the level of myelencephalon, small otocyst and unidentified eye primordia (anophthalmia), at the level of abdominal cavity which showed opening of neural tube and ectopia cordis with small size, thinned wall atrium and ventricle and unfused endocardial cushion, at the level of abdominal cavity which showed spinal cord which appeared as small and rudimentary structure and sank deeply into the dorsal body wall, small stomach and liver with hepatic cord and hepatic sinusoid, and the level of

mesonephros showed small and abnormal spinal cord, large cystic cavity in mesonephros, very small number of mesonephric tubules and caudal degeneration was indicated in this embryo.

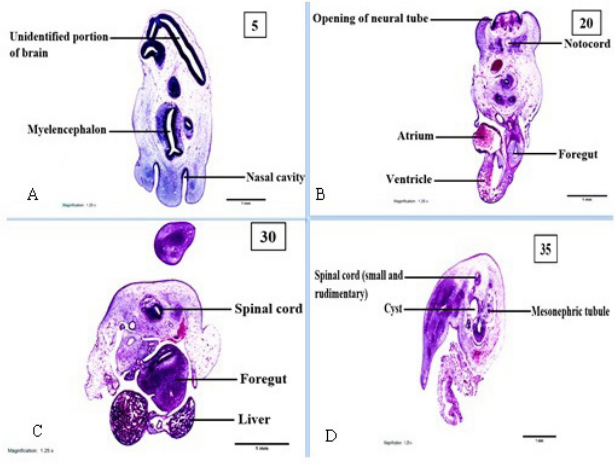


Fig 7. A, B, C, D were serial section of day 6 chick embryo injected with 1.5 mg of MSG, section number 5, 20, 30 and 35, respectively to show multiple malformations in the same embryo. Fig 7A and 7B were at the head region to show irregular shaped of brain. Fig 7B showed the widely opened of the neural tube dorsally with ectopia cordis ventrally. Fig 7C showed small stomach and liver situated outside the body cavity. Fig 7D showed small and under developed mesonephros. The brain and spinal cord were irregularly-shaped, the spinal cord was small and rudimentary. This embryo showed anencephaly, anophthalmia, spina bifida, ectopia cordis and under developed visceral organs and ectopia viscerae.

3. Dose 2.5 mg of MSG/gm egg weight (Fig 8)

The serial section of dose 2.5 mg of MSG/gm egg weight showed opening anterior neuropore and unidentified portion of brain at the level of approximately diencephalon showed no primordial of both eyes called anophthalmia, at the level of approximately myelencephalon showed small otocyst, acousticofacial ganglion and stomodeum, at the level of mesonephros and gonad showed small number of mesonephric tubules, ectopiacordis, underdevelopment with thinned wall atrium and ventricle without endocardial cushion, abnormal spinal cord and ectopia viscera of liver at the relation of ventricle and opened posterior neuropore were also indicated.

4. Dose 3 mg of MSG/gm egg weight (Fig 9)

The serial section of dose 3 mg of MSG/gm egg weight showed severe abnormality of neural development. The rachischisis of the neural tube was at the level of brain. The spinal cord showed double spinal cords with double notochords. There was underdevelopment of stomach. There was ectopia cordis with heart valve, abnormality

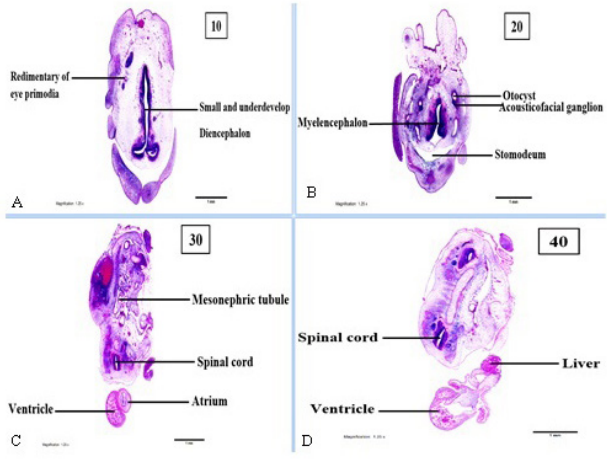


Fig 8. A, B, C, D were serial section of day 6 chick embryo injected with 2.5 mg of MSG, section number 10, 20, 30 and 40, respectively to show multiple malformations in the same embryo. Fig 8A and 8B were at the head region to show irregular shape brain with no eye primordium. Fig 8B showed irregular myelencephalon with small otocyst. Fig 8C showed small spinal cord and viscerae situated inside the body cavity with the heart situated outside, small and under developed mesonephros situated inside the body cavity. Fig 8D showed spinal cord was small and rudimentary. This embryo showed anencephaly, anophthalmia, abnormal spinal cord, ectopia cordis and uner developed visceral organs.

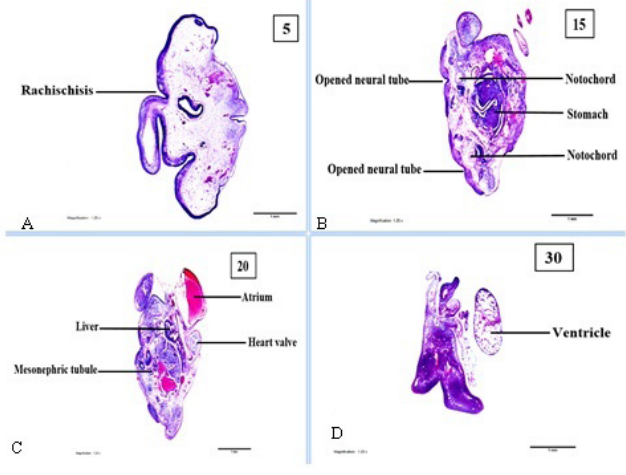


Fig 9. A, B, C, D were serial section of day 6 chick embryo injected with 3 mg of MSG, section number 5, 15, 20 and 30, respectively to show multiple malformations in the same embryo. This embryo showed opening of neural tube for the whole length of the body (rachischsis), Fig 9A and 9B were at the head region to show irregular shaped of brain, Fig 9B showed the wide opened of anterior neuropore, and without eye primordium. Fig 9C showed small viscerae within the body cavity and the heart situated outside (ectopiacordis). Fig 9D showed small and under developed mesonephros. This embryo showed rachischisis, anophthalmia, ectopiacordis and under developed of all visceral organs.

and large cystic cavity of atrium. There was dilated liver cord and liver sinusoid and very small number of mesonephric tubules of mesonephros.

There were common types of malformation which occurred in the treated groups of all concentrations. The abnormalities comprised anencephaly, rachischisis, spina bifida, anophthalmia, microphthalmia, ectopia cordis, ectopia viscerae, underdeveloped heart and all viscerae.

All survival day 10 embryos showed normal development except one of the 3 mg injection group showed ectopia viscerae. All parameters measured showed no significant difference when compared with the control. The dead embryos were less than 50% in all groups.

DISCUSSION

Several studies reported in animal models and humans that MSG effected central nervous system, reproductive system, adipose tissue and liver.[11] The effect of MSG in this study showed that the mortality rate increased in higher doses while the abnormalities were identified in all doses, which corresponded with the incidence of death and malformation rate in pregnant rats that received MSG.[13]

The effects of MSG on the brain were small size, opened anterior neuropore (anencephaly) and the brain vesicle was not completed on day 3 chick embryos in dose 1.5 mg and 3 mg of MSG/gm egg weight. The day 6 chick embryos showed opened of anterior neuropore and unidentified part of brain in dose 1 mg, 1.5 mg and 2.5 mg of MSG/gm egg weight and rachischisis in dose 3 mg of MSG/gm egg weight. The results agreed with the study of Jurand, in 1973 who studied the teratogenic activity of methadone hydrochloride in mouse and chick embryos and showed that the anterior neuropore was opened with rachischisis of brain.[12] Moreover, in 2009, Schmidt *et al.,* reported maternal caffeine consumption showed increased risk of NTDs and anencephaly was also observed.[13]

The effect of MSG on the eye development revealed both retardation and abnormality. The retardation was indicated by the persistence of intra retinal space and lens pit. The abnormalities included microphthalmos and anophthalmos. This result agreed with Qudsi *et al.,* who studied the effect of monosodium glutamate on chick embryo which also revealed monophthalmia.[1] In 2014, Zheng *et al.,* studied excess caffeine exposure which impaired eye development during chick embryogenesis and reported it caused asymmetrical micropthalmia by increasing ROS production and perturbed Pax6 expression due to the effect of high salt-exposure on the development of retina and lens.

The effect of MSG on the heart development showed elongated heart tube and U-shaped heart looping, thinned wall and dilated lumen on day 3 chick embryos. This study corresponding with the study of Abdelkader *et al.,* about teratogenicity and brain aromatase-induction of monosodium glutamate in estrogen-responsive mosaic transgenic zebra fish *Daniorerio* showed elongated heart and pericardial edema.[14] In 2016, Mahaliyana *et al.,* studied the effects of MSG on embryonic heart development of zebrafish (*Daniorerio*), a promising animal model, and demonstrated internal anatomical deformities such as elongated heart, cardiac sac edema and yolk-sac edema.

The effect of MSG on chick embryos to the anterior body wall showed ectopia viscera with ectopia cordis in all doses such as 1 mg, 1.5 mg, 2.5 mg and 3 mg of MSG/gm egg weight observed by the external morphology and the serial section of day 6, 10 chick embryos. The result corresponded with the study of Qudsi *et al.,* who studied the effect of MSG on chick embryo development which showed different congenital malformations in treated chick embryos such as abdominal hernia, bleeding, monophthalmia and brain deformation.[1]

CONCLUSION

The striking abnormalities caused by MSG were NTDs, brain showed irregularly shape and unidentified portion, widely opened of anterior neuropore, and severe opening of the neural tube or rachischisis of brain and spinal cord. Some embryos showed opening of the posterior neuropore. Eyes were also the target organ of the MSG as there were several types of abnormality, microphthalmia and anophthalmia, retardation of eye development indicated by persistent of intraretinal space and lens pit. The heart showed ectopiacordis, underdeveloped with thinned wall atrium and ventricle and unfused endocardial cushion. The visceral organs were totally underdeveloped. The mesonephros was smaller than the control and showed abnormal development with lesser number of mesonephric tubules and bilateral large cystic cavities. The gonads were underdeveloped with smaller size of gonadal ridges. This experiment clearly indicated that MSG was a potent teratogen. Therefore, it is essential to make people aware of the effects of MSG, especially for the pregnant women who should avoid consumption altogether.

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Rituximab Treatment for Paraneoplastic Pemphigus Associated with Follicular B-Cell Lymphoma: A Case Report and Review of Treatment Outcome

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ABSTRACT

Objective: To report a case of paraneoplastic pemphigus (PNP), a rare disease, associated with follicular B-cell lymphoma. Treatment outcomes of PNP associated with follicular B-cell lymphoma following rituximab treatment were also reviewed in this article.

Case presentation: We reported a PNP patient presenting with intractable stomatitis and erythema multiforme-like lesions. Skin biopsy from erythema multiforme-liked lesion revealed interface dermatitis and necrotic keratinocytes. Direct immunofluorescence demonstrated immunoglobulin G deposition at intercellular space, as well as complement deposition at dermo-epidermal junction. Indirect immunofluorescence using rat bladder substrate was helpful in confirming the diagnosis of PNP. Further investigations revealed an underlying disease of follicular B-cell lymphoma. He was treated with rituximab and systemic corticosteroids. Improvement of mucocutaneous lesions and lymphoma were noted. Nevertheless, he developed hospital-acquired pneumonia and died from septic shock shortly after receiving conventional chemotherapy. His survival duration was approximately 8 months after diagnosis of PNP.

Conclusion: It seemed that rituximab might provide promising benefits for mucocutaneous lesions in PNP patients associated with follicular B-cell lymphoma.

Keywords: Paraneoplastic pemphigus; rituximab; follicular B-cell lymphoma; therapy; bronchiolitis obliterans (Siriraj Med J 2018;70: 523-528)

INTRODUCTION

Paraneoplastic pemphigus (PNP), a rare disease, is characterized by severe intractable stomatitis and polymorphous skin eruptions in patients with underlying neoplasms.[1] Treatment of neoplasm is the most important, but variable results for mucocutaneous lesions have been demonstrated.[2] To control mucocutaneous lesions, high dose corticosteroids and immunosuppressive drugs are frequently used which can lead to severe infections and other complications. Recently, rituximab (anti-CD20 antibodies) has been used in PNP patients with CD20+ lymphoma.[3-5] Herein, we reported our case and review treatment outcome of rituximab in PNP patients associated with follicular B-cell lymphoma.

CASE REPORT

A 44-year-old man without any underlying disease came to see a rheumatologist due to chronic stomatitis and weight loss of 18 kilograms for 6 months. Prednisolone 20 mg/day, hydroxychloroquine 200 mg/day and colchicine

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0.6 mg/day were initiated due to a suspicion of Bechet's disease. After 2 weeks of treatment, a patient had a worsening of his oral lesion and developed erosive erythematous patches and target-like lesions on his genitalia, trunk, and extremities (Fig 1). He was then referred for dermatologic consultation. Steven-Johnsons syndrome and PNP were suspected due to stomatitis and erythema multiforme-like lesions. Possible drugs including colchicine and hydroxychloroquine were discontinued. After stopping the possible drugs for 2 weeks, his mucocutaneous lesions still deteriorated. He developed mild dyspnea and dry cough. Skin biopsy from erythema multiforme-liked lesion revealed interface dermatitis and necrotic keratinocytes. Direct immunofluorescence demonstrated immunoglobulin G deposition at intercellular space, as well as complement deposition at dermo-epidermal junction. Indirect immunofluorescence was positive for rat bladder substrate (Fig 1a-1d). The diagnosis of PNP was established and oral prednisolone 60 mg/day (1 mg/kg/day) was started to control mucocutaneous lesions.

Computed tomography (CT) angiogram of the chest demonstrated diffuse peribronchial wall thickening with bronchiolitis, possibly lymphadenopathy along periaortic region, and a focal ground glass opacity at upper lobe of right lung. CT-guided biopsy showed small to medium sized atypical lymphoid cells with positive immunostaining of CD3, CD20, CD10, CD43 and bcl-2, but negative immunostaining for CD5, CD23, or cyclin D1. For disease staging, abdominal CT scan was performed which revealed enlargement of intraabdominal nodes including paraaortic, retrocaval, aortocaval, common iliac, and celiac-mesenteric regions. Bone marrow biopsy showed no invasion of atypical lymphoid cells. These findings were compatible with follicular B-cell lymphoma stage II. Bronchoscopy and bronchoalveolar lavage (BAL) were undergone due to fever, dyspnea, and abnormal chest imaging at upper lobe of right lung. Polymerase chain reaction and culture of BAL fluid for *Mycobacterium tuberculosis* were negative. However, anti-tuberculous drugs were initiated because smear negative pulmonary tuberculosis could not be excluded. Moreover, antifungal drugs were commenced as BAL fluid revealed positive for galactomannan antigen. To prevent adrenal crisis and control severe infections, prednisolone was gradually decreased from 60 mg to 15 mg resulting in deterioration in mucocutaneous lesions.

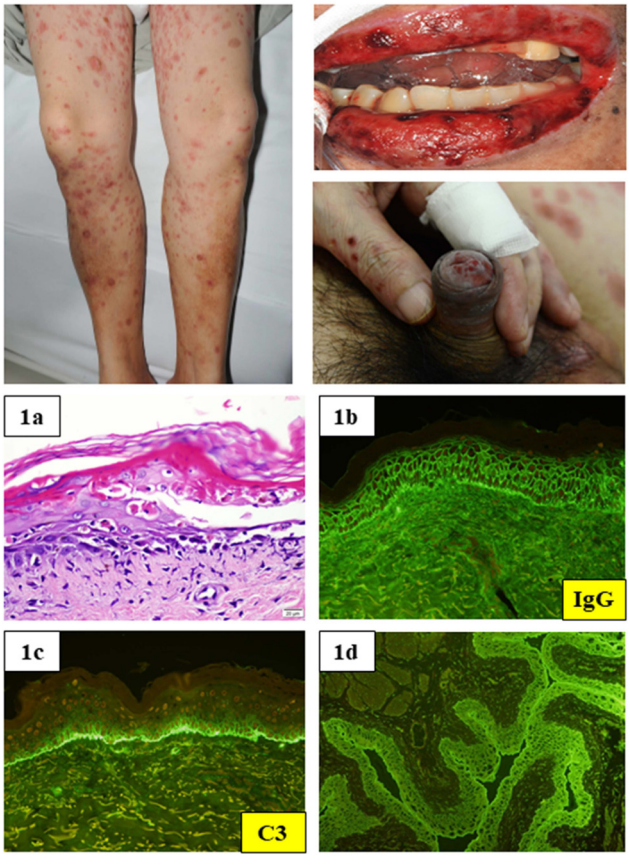


Fig 1. Clinical presentations, histopathology, and immunofluorescent results

One month after tuberculosis and fungal treatment, serious infections were controllable. However, rituximab-based chemotherapy for follicular B-cell lymphoma could not be initiated due to his performance status. Thus, rituximab at the dose of 375 mg/m^2 with prednisolone 45 mg/day every 21-day was used to control both PNP and follicular B-cell lymphoma. After a third cycle of rituximab there was an improvement in mucocutaneous lesions (Fig 2) and CT chest showed a decrease in the size of mediastinal and hilar lymph nodes. However, he had an acute exacerbation of chronic dyspnea after the fifth cycle of rituximab. A repeated CT chest showed pleuroparenchymal fibrosis which led to the suspicion of bronchiolitis obliterans. Nevertheless, lung function test was not able to be performed due to very poor performance status of the patient. After the 8th cycle of rituximab, he received one cycle of CVP (cyclophosphamide, vincristine, prednisolone) regimen. Shortly afterwards, the patient developed hospital-acquired pneumonia and died from septic shock. His survival duration after diagnosis of PNP with follicular B-cell lymphoma was approximately 8 months.

DISCUSSION

Due to a wide range of clinical presentations and complexity in the pathogenesis, the diagnosis and treatment of PNP remains a great challenge. Similar to our patient, severe painful, hemorrhagic, therapy-resistant stomatitis is the earliest complaint and usually precedes cutaneous lesions.[6] The retrospective study in 12 Korean patients with



Fig 2. Mucocutaneous lesions (a) before rituximab initiation, (b) a 3rd cycle of rituximab, (c) a 5th cycle of rituximab

PNP showed that erythema multiforme-like or lichenoid eruptions were more common than blister-like lesion in the East Asian population.[7] Bronchiolitis obliterans, a rare and fatal complication, is characterized by progressive obliteration of small airways and a persistent decline in lung function. The prognosis of PNP is very poor with a mortality rate of 90%. Common causes of death are sepsis, respiratory failure, and underlying neoplasm.[1,2,6]

Corticosteroids are the first-line treatment of mucocutaneous lesions. Steroid sparing agents such as azathioprine, cyclophosphamide, cyclosporine, and mycophenolate mofetil or autoantibody-lowering agents (intravenous immunoglobulin and plasmapheresis) have been used with variable results.[2] Based on our literature review, rituximab has been used in some PNP patients with follicular B-cell lymphoma since 2001. Table 1 shows demographic data and outcomes of reported cases of PNP-related follicular lymphoma patients receiving rituximab treatment.[3-5,9-18] It was shown that neither conventional chemotherapy nor steroid sparing agent yielded satisfactory results in mucocutaneous lesions. On the other hand, rituximab seemed to induce a favorable outcome. Twelve of 14 patients (85.7%), including our patient, had an improvement in mucocutaneous lesions (Table 1).

Bronchiolitis obliterans, an irreversible obstructive lung disease, is a major cause of death in PNP patients.[6], Lee *et al.*, reported a patient who achieved the longest survival (27 months) of PNP-related bronchiolitis obliterans from R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone) regimen.[18] However, Lee *et al.*, stated that full dose of chemotherapeutic regimen, not only rituximab, led to this promising result.[18] Some authors stated that rituximab in combination with cyclosporine is one of a few treatment options for PNP that can lead to long-term remission.[19,20] Up to date, it is still difficult to determine a treatment protocol and treatment outcome of rituximab in PNP due to a low prevalence of the disease.[1,2]

Conversely, several protocols including a lymphoma protocol, a rheumatoid arthritis protocol, low-dose rituximab with or without concomitant use of intravenous immunoglobulin and immunoadsorption have been proposed in treating pemphigus vulgaris or pemphigus foliaceous patients. Current evidence suggests that rituximab has a favourable outcome in treating patients with pemphigus vulgaris and pemphigus foliaceous.[21] Complete and clinical remission rates were approximately 65-78%. Mean time to disease control was less than one month. The underlying neoplasm of PNP, rather than an autoimmune process, may be a reason for the difference in clinical outcomes following rituximab treatment between PNP and other variants of pemphigus.[21]

With regards to the patient's primary disease, rituximab seems to change the paradigm of treating follicular B cell lymphoma.[22] Several randomized trials demonstrated improved response rates time to progression and overall survival rate in the rituximab plus chemotherapy patients.[23-27] Rituximab was shown to rapidly deplete CD20+ tumor B-cells and the effect was detected after a few months of treatment.[28] Moreover, using rituximab as a maintenance therapy in patients who already received rituximab-based chemotherapy showed higher disease-free duration.[29]

The most common reported adverse events of rituximab were infusion reactions.[30] Many reactions are dose-dependent and developed within 24 hours of the first infusion. Slow infusion rate, premedication with antihistamine, acetaminophen, and/or corticosteroids can reduce symptoms in non-severe reactions. In patients with severe reactions, infusion would be permanently withdrawn. A minimal risk to develop leukopenia and infection in rituximab maintenance therapy has been reported.[31]

In conclusion, due to PNP is a relatively rare disease, it is difficult to determine the efficacy of rituximab in PNP associated with lymphoproliferative disorders or other neoplasms. Based on limited evidence, it seemed that rituximab may provide promising results of mucocutaneous lesions in PNP associated follicular B-cell lymphoma.

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TABLE 1. Reported cases of paraneoplastic pemphigus (PNP) with follicular lymphoma treated with rituximab

Studies	Year	Age (yrs) / Sex	Lymphoma staging	First treatment of PNP and lymphoma	Outcome from previous treatment	Rituximab(R) and other treatment	Mucocutaneous lesions after rituximab treatment	Follow-up time
Borradori [9]	2001	61 / F	IVA	Prednisolone and 6 cycles of CHOP regimen	Slightly improvement in stomatitis	R: 375 mg/m^2 weekly × 4 weeks then every 2 months	1 month: rapidly improved	4 months
Heizmann [3]	2001	73 / F	Not reported	Prednisolone and cyclophosphamide for 4 weeks	No improvement in mucocutaneous lesions	R: 375 mg/m^2 weekly × 4 weeks	1 week: regression of lesions 6 months: completely resolved 1 year: still absent	1 year
Bernadas [4]	2006	77 / F	IIB	Prednisolone and cyclosporine	No improvement in mucocutaneous lesions and lymph nodes	R: 375 mg/m^2 weekly × 4 weeks and methylprednisolone	8 months: completely resolved	3 years: death
Hoque [10]	2006	49 / F	Not reported	-	-	R-CVP chemotherapy × 3 cycles	1 cycle: improved in the rash but briefly recurred	Death
Deborah [11]	2007	71 / F	Not reported	4 cycles of CHOP	Disease remission but briefly recurred	R: 375 mg/m^2 weekly × 4 weeks and this 4-dose was repeated every 6 months, daclizumab, prednisolone	During 2 years of follow-up: less active of mucositis	2 years
Aoi [5]	2013	60 / M	Not reported	Prednisolone	No improvement in mucocutaneous lesions	R: 375 mg/m^2 weekly × 8 weeks and chemotherapy	5 weeks: almost recovery	Not reported
Morikawa [12]	2014	65 / F	IIIA	-	-	R-CHOP, CVP chemotherapy	Improvement in mucocutaneous lesions	5 months
Hirano [13]	2015	60 / M	Not reported	-	-	R: 375 mg/m^2 weekly × 8 weeks	8 weeks: completely resolved of blisters	14 months: death

Abbreviations: CHOP; cyclophosphamide, doxorubicin, vincristine, prednisolone, CVP; cyclophosphamide, vincristine, prednisolone, IVIG; intravenous immunoglobulin, R; Rituximab

TABLE 1. Reported cases of paraneoplastic pemphigus (PNP) with follicular lymphoma treated with rituximab

Studies	Year	Age (yrs) / Sex	Lymphoma staging	First treatment of PNP and lymphoma	Outcome from previous treatment	Rituximab(R) and other treatment	Mucocutaneous lesions after rituximab treatment	Follow-up time
Rossom [14]	2015	70 / M	IVB	Di-Adreson-F aquosum, cyclophosphamide, azathioprine	No improvement	R: 375 mg/m^2 weekly × 4 weeks and prednisolone	8 weeks: slightly improved	9 months: death from heart failure
Kanwar [15]	2015	61 / M	Not reported	Prednisolone	Poor control	R: 375 mg/m^2 every 21 days × 4 cycles and prednisolone	4 cycles: all mucocutaneous lesions healed	3 months
Namba [16]	2016	59 / F	Not reported	Prednisolone and cyclosporine	N/A	R-CHOP × 2 doses, additional 5 cycles of R-375 mg/m^2, prednisolone, IVIG, cyclosporine	No improvement of mucosal lesions	6 months: death
Kikushi [17]	2017	74 / F	Histological grade II	Prednisolone	No improvement	R: 375 mg/m^2 + Bendamustine	2 weeks: began to improve 4 weeks: almost resolved	1 year: no recurrence of PNP
Lee [18]	2017	53 / M	IVA	-	-	7 cycles of R-CHOP regimen and one additional cycle of R-375 mg/m^2	No improvement in mucocutaneous lesions but not progress	27 months: death from respiratory failure
Our case		44 / M	II	-	-	R: 375 mg/m^2 every 21 days × 8 cycles and prednisolone	Improvement in mucocutaneous lesions	8 months: death from septic shock

Abbreviations: CHOP; cyclophosphamide, doxorubicin, vincristine, prednisolone, CVP; cyclophosphamide, vincristine, prednisolone, IVIG; intravenous immunoglobulin, R; Rituximab

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The New Biomedicine: A Critical Appraisal

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ABSTRACT

Mahidol University's namesake, H.R.H. Prince Mahidol, stated the University's universal view of higher education as follows: 'True success is not in the learning, but in its application to the benefit of mankind'.[1] It is thus fitting that the following commentary speaks to the University's goal. The acquisition of basic scientific knowledge by scientists working in genetics, embryology, developmental biology, immunology and virology throughout the twentieth century led to its application to clinical problems in the twenty-first. The technology to repair genetically-deficient pluripotent stem cells, treating diseased adults or ensuring the birth of healthy babies now is almost within our power. However, the application of this knowledge to living organisms, using cells that can mutate and selectively evolve, makes clinical application tricky while social and ethical issues arising from the eventual use of these technologies requires thought. Future attention to trends in basic science research should make stem cell therapy applicable to all.

Keywords: Biomedicine; critical appraisal (Siriraj Med J 2018;70: 529-539)

PRELUDE

Arguably, the most important basic science development of relevance to the pluripotent stem cell field in the twentieth century was Leroy Stevens' description of mouse teratocarcinoma[2], tumors composed of an infinite number of differentiated cell types and tissues but also containing nests of undifferentiated, "embryonal" carcinoma cells. His sequentially, animal-passaged teratocarcinomas gave access to an experimental test of the cancer stem cell hypothesis. Indeed, injection of single embryonal carcinoma cells (ECC) into mice give rise to tumors containing the spectrum of cell types found in the original teratocarcinoma.[3] Cell lines derived from both mouse[4] and human teratocarcinomas [5] were derived, providing an even more accessible platform to dissect the characteristics of pluripotency.[6] In addition, the quest to determine the normal pluripotent stem cell (PSC)-equivalent to the ECC, was accomplished by transplanting day 3 to 6 day-old mouse embryos into ectopic sites in syngeneic mice, analyzing the tumors arising for the pluripotent phenotype. Such transplants gave rise to teratomas containing the characteristic complexity of differentiated cell types.[7,8] This finding encouraged. the derivation and characterization of mouse embryonic stem cell lines (mESCs) in cell culture[9,10] Some years later human embryonic stem cells (hESCs)[11] were isolated and the ability of multiple hESCs cell lines to differentiate into the multiple human differentiated cell types was characterized.[11]

In parallel with these findings the search for replacement tissue and cell therapies in medicine passed from organ, tissue and cell transplantation in animal

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models to clinical trials. As the principal immunologic requirements for successful transplantation and their control evolved, the first human corneal, kidney, heart and liver transplants, as well as replacement of the whole or parts of the hematopoietic system took place. That epithelial cells cultured *in vitro*, could be grown and expanded *in vivo* led to the idea that specific cell types could be isolated and quickly expanded in quantities useful for transplantation. Pioneering work to this effect was first performed by isolating and growing patient's epidermal keratinocytes and then using them to provide autologous transplants to provide coverage for burn victims.[12] The availability of PSCs further expanded the possibility to provide, through their differentiation other progenitor, precursor or differentiated cell type for clinical use.

Concomitantly, the emergence of DNA sequencing and its application to gene editing by homologous recombination[13,14] changed genetics from observational to experimental branch of science. This rapid evolution led to an understanding of the phenotypic consequences of dysfunctional genes presaging the first efforts towards gene replacement therapy in humans to effect cures. Indeed, the latter years of the 20th century became a hot bed for correcting genes first *in vitro*, then in animal models *in vivo*, and then translating these advances for clinical use.

The first reported gene therapy clinical trials, reported before the turn of the century, involved transgenesis of two adenosine deaminase (ADA)-deficient severe combined immune-deficient (SCID) patients. In the first study they were transfused with their own peripheral blood T-lymphocytes (PBL) which had been transfected *in vitro* to Moloney murine leukemia virus (MoMLV) driving a recombinant ADA cDNA.[15] Four years after the transfusions took place the patient's ADA levels were reported to be normal, however these patients were also being treated with pegylated ADA. Others, also using *in vitro* transfection of an MoMLV-ADAcDNA vector into the patient's PBLs and bone marrow-derived cells and subsequent transfusion, reported short term reconstitution after transfusion of either cell type but only bone marrow progenitor cells provided long term reconstitution.[16] Again these patients were also continuously treated with pegylated human ADA, which muddied the results. Another very early clinical trial in a completely different organ system, reported a very small, persistent dimunition of lipoprotein levels in a hypercholesterolaemic patient. The patient was partially hepatectomized, his dissociated liver cells, exposed to a recombinant MoMLV-chicken actin-promoted human LDL receptor-cDNA vector *in vitro* and his cells were then transfused back to autologous, partially hepatectomized, enzyme-deficient host.[17] On the face of it these trials established the relative safety of vector-mediated gene therapy and hinted at the efficacy of transducing long term progenitor cells.

Regenerative Medicine in the Twenty First Century Genetic Engineering

Follow-ups to these Phase 1/2 clincal trials produced initially promising results. Two studies were initiated. $CD4^+$ bone marrow-derived cells, transduced with a replication-defective recombinant MoMLV retrovirus containing the relevant human transgene, were transplanted into X-linked SCID and ADA-SCID patients. Long-term and sufficient levels of transgene expression were reported.[18,19] However, after some time patients in these and other similar trials presented with leukemias, a predictable consequence of viral vector integration into active host genes. Retroviral integration into oncogenes leads to their overexpression, resulting in uncontrolled growth of these cells and subsequent progession to leukemia.[20,21] Around the same time a pilot study to study the effect of an intravascular dose of a liver-directed adenovirus-5 vector containing ornithine transcarbamylase (*OTC*) to a partially OTC-deficient patient also produced disturbing results. The enzyme-deficient patient had an acute, uncontrollable inflammatory response to the adeno-5 viral vector and died.[22] Accordingly, a recess on viral-directed gene therapy was declared to allow time for vector biology/pharmacology to be addressed and to explore other methods to supply enzymes and other gene products to ailing human hosts.

The efficiency of genome editing improved steadily in the early part of this century. First, zinc finger proteins (ZFNs) were used to successfully target the specific gene recognition of DNA nucleases. These targeted nucleases could induce double strand breaks at specific sites in the genome and proper gene repair by homologous recombination was ensured by introducing a vector-born gene template for successful repair.[23,24] The transcription activator-like effector nucleases (TALENs) provided a more cost-effect way to mediate gene recognition by the DNA nucleases[25,26] and were also applied to human gene correction. However, the discovery of the bacterial defense system against bacteriophages, i.e. clustered regularly interspaced short palindromic repeats (CRISPR) , which uses the CRISPR-associated nuclease (Cas9) targeted to the genome by short RNA guides[27], opened mammalian genome repair to a wide range of scientists because of the simplicity of targeting and ease of operartion.[28,29] Corrective repair of the nick in the genome is repaired by

host-induced non-homologous end joining or, if a DNA template is furnished, by homologous recombination. The basic science discoveries underlying this fast moving field have been recently reviewed.[30]

The clinical use of viral vectors for gene therapy has also evolved. Due to their ease of delivery and their ability to infect a broad range of dividing and nondividing cells the adenoviral and recombinant adeno-associated viral vectors (rAAV) have been favored. However, because of the possibility of a strong immune response to the adenoviral vectors their use is currently somewhat limited mainly to oncolytic tumor therapies or to therapies in immunologically privileged sites. rAAV vectors, which can integrate into the host genome but are primarily episomal vectors, are now being actively used to transduce single human genes therapeutically.[31] Indeed, intravenous delivery of a chicken b-actin promoted-survival motor neuron1 (SMN1) transgene, delivered in an rAAV vector, resulted in improvement in the survival and motor function of fifteen young children diagnosed with spinal muscular atrophy, who were screened at the outset of treatment and found free of antibodies to AAV serotype 9. However, most of these patients eventually did develop elevated liver enzymes, perhaps as a result of the immune response to AAV, which returned to normal levels after prednisolone treatment.[32] A subsequent report of elevated liver enzymes and also neuronal toxicity following high level of AAV/SMN1 administration to nonhuman primates and piglets[33] predicts this viral vector will also prove unsafe for future therapeutic purposes. When long term expression via integration into a largely non-dividing cell population is required the g-retroviral vectors (largely MoMULV-based) cannot be used, however, modified lentiviral vectors can transduce genes into both dividing and nondividing cells. Current lentiviral vectors take advantage of the broad tropism of the pseudotyped VSV glycoprotein so they are versatile, albeit difficult to manufacture under the GMP-conditions necessary for the high titers needed for gene therapy.[34] The reader is directed to a comprehensive review on emerging themes in gene therapy, targeting various organ systems, by the currently used viral vectors[22]

Correction of host genes has now been directly effected *in vivo*. A phase 3 clinical trial demonstrated the clinical efficacy of the FDA-approved drug of Nursinerin, a mixture of 18-mer oligonucleotides, designed to activate the paralogous SMN2 gene, by rearranged splicing, to produce SMN1 protein. Infants who had been previously diagnosed with SMA were treated by multiple Nursinerin injections, directly into their cerebral spinal fluids. Forty-one percent of the treatment group and none of the control group reached their motor milestone responses a year after the initiation of the trial.[35] The next quest will be to identify SMN1 mutations at birth, start Nursinerin treatment immediately and determine whether the entire dSMA disease entity can be prevented.

Successful therapy by direct gene editing has recently been accomplished in Beethoven mice, a mouse model of human deafness resulting from mutation of a gene component of the mechano-transduction channels in mammalian hair cells. A mixture of Lipofectamine 2000 and a Cas9 nuclease-guideRNA ribonucleprotein complex were delivered by direct injection into the cochlea. A therapeutic effect was noted, measured by acoustic startle responses, in the treated, versus the untreated, ear of the same animals.[36] The potential of off-target effects, found to be negligible in this example, is always an area of concern for *in vivo* gene repair therapies. In addition, the possibility of antibodies to the bacterial CAS9 nuclease in human subjects has been raised and is now under investigation.[37] The efficacy of gene therapy, be it by supply or correction, has survived the proof of principle in animal models and clinical medicine but a safe, reliable delivery system requires further investigation. Perhaps nanoparticle or lipid-based delivery systems currently in development will prove effective. Nonetheless, deafness has been a long-term social problem in our ever enlarging aging populations, comprised of individuals often willing to enter into clinical trials, so this may well be the next frontier for gene correction therapy.

Stem Cell Therapy

In 2006, Shinya Yamanaka and his colleagues accomplished an experimental breakthrough, making use of a technique that targets transcription factor expression within a given cell type to induce its transdifferentiation into a different one.[38] They showed that forced expression of a combination of relevant transcription factors (OCT4, SOX2, KLF4 and MYC) in differentiated mouse[39] or human[40] cells could induce them to pluripotency. These induced-pluripotent stem cells (iPSC), like embryo-derived mouse and human ESCs, were capable of differentiating into essentially all cell types of the adult organism when differentiated via an *in vitro*, embryoid body assay or via an *in vivo*, teratoma assay in immundeficient mice. iPSCs, reprogrammed *in vitro*, may differ epigenetically from ESCs, derived from embryos that have undergone natural reprogramming *in vivo*. Most genomic methylation marks are flexibly reconstituted however, the methylation of imprinted genes may be at issue, setting up potential differences between ESCs and iPSCs. Whether any epigenetic differences remain between these two types of PSC and

whether more genetic perturbations occur because of this additional manipulation is under investigation but no global differences specific to one or the other cell type have been found.[41]

As soon as PSCs were isolated *in vitro* they were regarded as integral components of regenerative medicine. The esistence of iPSCs brought the concept of individualized regenerative medicine. One's own cells could be isolated, reprogrammed to pluripotency, then differentiated into suitable precursor, progenitor, or differentiated cells for autologous transplantation to treat diseases or traumatic injuries, without fear of immune rejection. Unfortunately, the possibility of cure by stem cells, be they pluri-, multi- or uni-potent, was seized upon by unscrupulous practitioners who marketed their use through clinics fraudulently claiming cures of various diseases. Because of this malpractice, individuals and the International Stem Society for Stem Cell Research called for governmental regulation of the field of regenerative medicine. Meanwhile, genuine clinical trials had been initiated with PSCs and the results are now being published in the scientific literature. As of November, 2017 the US Food and Drug Administration has the oversight of the use of human stem cells as drugs. The World Health Organization has been asked to collaborate with international groups active in cell therapy with the goal of providing guidelines to promote the development of safe and effective gene therapies within an international regulatory framework; a role that has yet to be effected.[42]

In 2003, the International Stem Cell Forum funded the International Stem Cell Initiative (ISCI) to define the human PSC by investigating their basic properties[6] and performing collaborative experiments related to their use in human medicine. PSCs proliferate (self-renew), they can differentiate, or they can die. Mutations occur in the genome of any living cell. If this mutation affects its robustness or proliferation it might grow selectively after any potentially disruptive event, e.g. ESC-isolation, iPSC-reprogramming, or even some random cell culture event, potentially conferring tumorigenic potential on its progeny. Unintentional malignancy is the worry of cell therapeutics.

For this reason the ISCI initiated a large study to compare 120 cell line pairs, from both early and late *in vitro* passage. The goal was to determine whether any common genomic changes appeared during their culture. It was already known that PSCs tended to accumulate particular chromosome abnormalities *in vitro*. Indeed, ~14% of the early passage cell lines and 33% of the high passage cell lines exhibited increased ploidy of chromosomes 1q, 12p, 17 and centromeric 20.[43] Further, single nucleotide polymorphism (SNP) analysis of the paired early and late passage cells revealed common amplification of an area of chromosome 20q11.21 in ~20% of the karyotypically normal cell pairs tested.[43] Analysis of the genes in the minimal 20q amplicon revealed *BCL2XL* as one of the three genes in this region that were expressed in PSCs. Overexpression of the BCL2L1 (BCL-XL isoform), and not the other two gene products, in normal PSCs conferred the rapid growth characteristics of the copy-number-variant PSCs, while inhibition of BCL-XL suppressed the PSCs growth advantage.[44] From these data BCL-XL was demonstrated to be a selected, potential driver mutation in some PSCs *in vitro*. Furthermore, most of the human embryonal carcinoma cell lines tested, and a human teratocarcinoma biopsy exhibited 20q11.21 amplification, circumstantially linking this mutation with malignant transformation[44]. A different large scale study of PSCs looked for and identified mutations in *TP53* in 14/257 cell lines tested.[45] Others exposed PSCs with p53 mutations to culture stress and found these cells exhibited increased cell proliferation, decreased apopotosis, decreased cell differentiation and enhanced clonogenic efficiency.[46]

Combine these studies show that mutations affecting two known drivers of human cancer, BCL-XL and p53, give rise to selective PSC growth *in vitro*. Whereas no known mutation is known to cause human teratocarcinoma (a common tumor in young men) studies of 129-strain mouse teratocarcinomas suggest a missed differentiation cue in some cells of the male embryonic germ line results in their continued proliferation and increases their opportunity for acquiring transformative mutations leading to testicular cancer.[47]

The ISCI also conducted a study to determine which of the commonly used *in vitro* or *in vivo* differentiation assays best predict PSC malignant potential. Only the teratoma assay, when analyzed by histology and immunohistochemistry to reveal nests embryonal carcinoma-like cells and yolk sac elements, or when analyzed by RNA-seq analysis and Teratoscore, to reveal molecular markers of pluripotency and yolk sac, was effective.[48] These findings suggest that random mutation and selective proliferation will be an underlying threat to PSCs as they are currently maintained. Although therapeutic transplants will focus on differentiated, or adult stem, cells derived from PSCs, the absence of malignant potential in the starting cell population is a requirement for the initial PSC cell line. The International Stem Cell Initiative has discharged its experimental mandate, having set standards for both the PSC field and quality control imperatives for International Stem Cell Banking. Now, twenty years after isolation of

the first human PSC, a Japanese pharmaceutical company (Sumitomo Dainippon) has opened to produce cells for regenerative medicine clinical trials. More such companies will follow to service the evolving PSC industry.

Evidence for the first in human clinical trials of PSC-derivatives are all based on extensive preclinical trials in multiple mammalian model organisms. Two well known companies, Asterias and International Stem Cell Corporation, have ongoing Phase 1/2 clinical trials using ESC-derived oligodendritic cells for spinal cord injury and ESC-derived neural stem cells for Parkinson's disease and they have done or acquired preliminary evidence for trials in other organ systems. In this ESC-based industry both the ESC, and the ESC-derive adult stem cells to be transferred have been subjected to exhaustive, long-term tumorigenicity testing. Progress updates about these trials can be found on these company's websites.

The first clinical trials of PSC-derived cells in regenerative medicine appropriately focused on the macula, the functional central area of the retina, which is known to degenerate in the eyes of some elderly people. The macula, is composed of retinal pigmented epithelial cells (RPE), brown pigmented cells of cobblestone morphology, that are easily seen/identified upon spontaneous differentiation of PSCs *in vitro*, and in PSC-generated teratomas. There are several advantages to clinical transplantation of these terminally differentiated cells: the back of the eye is surgically approachable, it is generally regarded as an immunologically privileged site and the the transplant itself is confined to an accessible non-vital organ. Addtionally, blind elderly people are willing to participate in trials that might improve their chances of independent living. One of the first proper clinical trials, using suspensions of cells from differentiated RPEs from an allogeneic embryonic stem cell (ESC) line reported the safety of the procedure but very mixed results on efficacy.[50] These authors suggested some of the adverse results they found might have been associated with the short-term immunosuppression they used. Consequently, after the iPSC technology became routine, fibroblasts from two patients with macular degeneration were reprogrammed to iPSCs. After differentiation, the sheets of patient-derived RPEs were subjected to intensive genomic and epigenomic scrutiny and tumorigenicity testing. Afterwards, a sheet of autologous cells was transplanted under the first patient's fovea in the absence of immuno-suppressants. Despite signs of neovascularization and maintenance of the graft up to 1 year post-transplant, there was neither diminishment nor major improvement of the patient's visual acuity. The second patient in this clinical trial cohort was not transplanted with his own iPSC; three copy number variants were identified in the differentiated RPE cell sheets that differed from his own cells prior to reprogramming and the cells were thought to represent a potential safety risk.[51] He subsequently received an allogeneic transplant, which was not successful, most probably due to a surgical problem rather than immune rejection. A third clinical trial, consists of a cohort of ten patients, each of whom received a patch of retinal pigment epithelium differentiated from an allogeneic, stable, human ESC, grown on a human vitronectin-coated polyester membrane. This composite, cell and membrane, was transferred to the fovea using a specially manufactured tool to patients treated with a short-term immune suppressive regime, but the transplants persist without long-term immunosuppression. The reported results from two of these ten patients at a year of engraftment indicate both safety and efficacy.[52] The remaining eight patients will be reported on in future. A fourth phase 1/2 clinical trial used RPEs differentiated from another ESC line, grown on a different, parylene, substrate and then transplanted to the fovea. Here at about a year of transplant one of five patients in the trial displayed improved vision, three patients experienced worse vision and there was no change in the vision of one patient.[53] Taken together, it appears the efficacy of macular replacement depends on the methodology used but each trial reported the macular transplants themselves safe. No difference between the success of autologous (iPSC-derived) or allogeneic (ESC-derived) transplants at this particular host site were found, albeit this is regarded as an immunologically privileged site. Also, only one was an autologous (iPSC-derived) transplant.

Each clinical trial requires extensive preclinical experience and preparation in order to obtain permission of the relevant oversight agencies. From an industrial standpoint ensuring the safety of a single PSC line for transplantation is expensive in time and money. Therefore, minimizing the number of cell lines to be permitted is particularly relevant to industrializing the processes and improving outcomes.[54,55] However, when regarding transplants to other sites in the body, especially to the hematopoietic system, immune rejection of HLA-mismatched transplants leadx to rejection. The immunologic imperative for autologous, iPSC-derived, transplants for corrective transplantation is currently neither cost nor time efficient. To minimize the potential future problem of finding a PSC with a suitable HLA match the Japanese have proposed banking PSCs with a series of population-common homozygous HLA haplotypes (typing for HLA-A,-B,-C -DRB1,-DQB1, -DPB1 and perhaps KIR), to be used to for transplantation. In such a scenario regional

cell banks would focus on collecting and characterizing iPSCs of the most common haplotypes in their region's population for future therapeutic use. The feasibility of this approach and the number of tolerable mismatches has been recently modeled by retrospective analysis of cord blood transplantation pairs in Japan.[56]

There are some accessible organ systems, such as the skin, where populations of adult stem cells were derived for autologous transplantation to correct a defect or replace a tissue lost to trauma or aging. Keratinocytes explanted from a not-yet-affected area of skin from a patient with junctional epidermolysis bullosa, a skin disease caused by *LAMB3* mutation, were isolated *in vitro*, transduced with a normal *LAMB3* cDNA under the control of the MoLV long terminal repeat. Corrected colonies were isolated, expanded and transplanted back to the patient. Because populations of gene-corrected cells in the explanted epidermis were marked by the multiple integration sites of the transgene the authors could trace the derivation of the various patches of new skin. Analysis of these mosaic skin patches revealed that "specific stems cells persist contributing to both renewal and repair by giving rise to pools of progenitors that persist for various periods of time, replenishing differentiated cells to make short term contributions to wound healing".[57] Although these epidermal stem cells were not derived from PSCs, but rather came directly from a population of adult stem cells[56], this work supports the concept that an entire organ system can be readily regenerated from autologous transplants if the proper population of gene-corrected stem cells can be identified. (As an aside transplanted skin can be readily surveyed over the life time of the individual for malignant nodules.)

Gene correction in hematopoietic cells, suitable for transplantation to patients with hematopoietic defects, has been under intense scrutiny for some time. Many consider therapy with cells corrected *ex vivo* and then transferred for permanent correction in vivo preferable to vector-driven gene replacement therapy *in vivo*, which may pose an inherent danger to the patient. In early studies, the b-globin gene, from patients with sickle cell anemia was corrected by homologous recombination in bone marrow-derived $CD34^+$ cells. Unfortunately, after transplanting the population of HSC/progenitor cells into humanized immune-compromised mice, only a limited level of corrected cells could be found, suggesting that the transplanted cells contained more mature progenitors rather than the hematopoietic stem cells (HSC) necessary for extensive repopulation *in vivo*.[59] High expression levels of the *IL2R* gene was obtained after homologous recombination in $CD34^+$ cells isolated from a SCIDX1 patient. Here, cells transplanted into humanized immunocompromised mice were functional but the authors speculate whether the limited number of HSCs and precursors obtained in vitro could ever repopulate the absent lymphoid lineages in a diseased human.[60]

If gene correction can be performed in iPSCs, which are then to be differentiated into the HSCs necessary for successful long-term engraftment, corrective transplantation becomes a simple matter of cell selection and efficacy testing. This is the current sticking point in hematopoietic stem cell therapy, regardless of the source of the gene corrected cell. CRISPR/Cas9 correction of genes in iPSCs from patients afflicted with various anemias has provided many laboratories with cells for *in vitro* disease modeling.[61,62] Unfortunately, the *in vitro* hematopoietic differentiation protocols in current usage for human iPSC preferentially induce primitive hemangiogenic differentiation rather than definitive, adult hematopoiesis. For this reason the safety and efficacy of gene-corrected cells capable of differentiating into mature elements of the hematopoietic system cannot be estimated. The transcription factors[63], the cytokines and signaling pathways[64] required for definitive human hematopoetic differentiation have now been defined, providing the basic research tools necessary to isolate the cell types capable of forming all elements of the mature definitive hematopoietic system. Experiments in mice suggest combined manipulation of transcription factors, signaling pathways and cytokines in a spatial and temporal fashion can yield the cells necessary for engraftment and lineage-specific, nonmalignant reconstitution simply using adult vascular endothelial cells as a starting point.[65] Regardless of the cell source, using patient-derived, gene-corrected hematopoietic cells for autologous transplantation eliminates the possibility of immune rejection, unless the corrected gene product is recognized as foreign, and also eliminates the possibility of graft versus host disease. Managing allotransplantation, although possible, complicates this aspect of modern biomedicine.

Defining the cell type for transplantation is of essence but the manufacturing process required for even testing the safety and cell numbers required for efficacy is also to be considered. Fine tuning the process itself, under GMP, animal product-free conditions requires: reprogramming (by chemicals, small molecules or transcription factors); editing and selecting the corrected targeted gene (by CRISPR/Cas9 technology); and differentiation, expansion and selection of the currently undefined long-term repopulating cell type for transplantation. Many aspects of process development have been addressed in the

context of therapeutic adoptive T-cell transfer. Here large numbers of patients cells are purified, modified and reinfused for cancer therapy.[66]

Effecting cures from selected, differentiated, characterized cell populations will be a giant step into the area of personalized medicine. This year the California Stem Cell Agency, which invests in stem cell-based therapies, has funded four California-based studies to target patients with severe sickle cell disease. With other funded studies in progress, this area is targeted for rapid development.

Genetic Engineering in Oocytes and Zygotes

Ever since development of homologous recombination technology[13,14] scientists have sought methods to mutate or modify genes by injecting relevant constructs directly into the oocyte or zygote. Such a direct approach would significantly shorten the time necessary to produce genetically modified animals although it is complicated by the inability to use the positive/negative selection crucial for selecting successful homologous recombinants.[67] The frequency of homologous recombination without selection, even when DNA constructs were injected directly into cells[68], is so low that such an approach in embryos was completely impractical.

All this changed with the advent of CRISPR-Cas technology which targets the relevant gene with a frequency of 10-20% or higher. In addition, it became possible to target several genes simultaneously[69] thus significantly facilitating the production of animals carrying multiple mutations and avoiding very time consuming and expensive breeding protocols. CRISPR-Cas did not conceptually change the mutagenesis approach to the study of gene function but made it much faster, cheaper and more reliable. Moreover, the high frequency of gene targeting made it possible for the first time to contemplate similar experiments using human eggs and zygotes. The first such study describing the effect of OCT4 mutation in early human development was recently published.[70] Here, loss of OCT4 affected human preimplantation development as has been previously shown in mice, though it seems that the disruption of development occurs earlier in human embryos. This conclusion is somewhat tentative since a control, using mouse zygotes injected with the similar constructs, was not included. One can envision further, similar studies using CRISPR-Cas to study the role of specific genes in human development, albeit the limitations posed by the available material and by the difficulties of performing extensive analysis will likely limit the extent of such an approach.

CRISPR-Cas technologies provide however another, never before available, opportunity namely targeted correction of disease-causing mutations directly in the zygote resulting in complete cure of a genetic disease. Several "proof of principle" results have been reported using mouse zygotes.[71,72] Zygotes from mice carrying a dominant mutation in the *Crygc* gene that causes cataract have been injected with Cas9 mRNA, a guide RNA and an oligonucleotide resulting in homology directed (HDR).[72] Approximately half of the newborn mice derived from injected zygotes showed successful targeting of the mutant allele while the wild type allele was not affected. Sequencing of the mutant alleles showed HDR as well as non-homologous end joining (NHEJ) repair and interestingly, in few of NHEJ cases the mutant phenotype was accidentally corrected. Similar results were obtained when attempting to correct *Dmd* gene, mutation of which results in mouse model of Duchenne muscular dystrophy (DMD).[71] Sequencing of the targeted allele showed the presence of both HDR and NHEJ and also a degree of genetic mosaicism in successfully targeted mice. The mosaicism is likely caused by the delay of CRSPR-Cas mediated targeting so that targeting occurs following one or two divisions thus affecting some but not all blastomeres of the cleaving embryo. Interestingly due to the specific mechanism of DMD even partial correction in mosaic animals resulted in significant phenotypic improvement.[71] These and other similar reports indicated that gene correction in the zygote using CRISPR-Cas is indeed possible but not without its problems. The frequency of successful targeting and correction was not 100% and mosaicism occurred relatively frequently which could have compromised the results, depending on the nature of the mutation. However, off target effects and targeting of the wild type allele were not commonm though they could not be excluded and most certainly could affect the use of this technology to correct human mutations.

The first attempt of gene targeting using human embryos was reported in 2015.[73] The authors used polyspermic, tripronuclear zygotes that are occasionally produced in fertility clinics following in vitro fertilization (IVF) and are normally discarded. They attempted targeting of the endogenous β-globin gene with mixed results. Though HDR was observed, the success rate was modest and embryos showing gene editing were mosaic. Off-target effects were also observed as well as NHEJ. Similar results were reported in an experiment attempting to introduce the naturally occurring *CCR5Δ32* allele (homozygous carriers of this allele are resistant to HIV infection). Here the authors observed a low incidence of HDR, mosaicism and possible off-target effects.[74]

Modification of the CRISPR-Cas technology, the so called "base editor" that can target and promote single base conversion, has recently been used to attempt correction of the mutation causing β-thalassemia.[75] The authors used cloned human embryos produced by enucleating human oocytesm fusing them with lymphocytes from a patient with β-thalassemia. Base editing, i.e. correction of the mutation, was observed in a relatively high number of analyzed blastomeres but mosaicism was always present. These studies though preliminary indicate that gene editing in human embryo is technically feasible though many problems remain to be solved before its use in the clinic can be contemplated.

Recently a very detailed study using both human zygotes and oocytes, attempting to correct the dominant mutation in the MYBPC3 gene that causes hypertrophic cardiomyopathy (HCM) has been published.[76] The mutation, which the authors attempted to correct, involves a four base deletion in exon 16 of the MYBPC3 gene. Sperm from a heterozygous patient with this mutation was used for IVF and either zygotes or M2 oocytes were injected with appropriate targeting constructs. In the case of M2 oocytes the targeting constructs were injected together with the sperm. Targeting constructs consisted of the Cas9 protein and guide RNA with or without single stranded donor oligonucleotide (ssODN) necessary for HDR. The authors suggested that injection of M2 oocytes eliminates mosaicism and that HDR can occur (as others have also occasionally reported) with high frequency even in embryos not injected with ssODN. This type of repair is supposedly mediated by the wild-type allele as a repair template. The report is rather optimistic indicating a high frequency (not 100%) of repair, a way of avoiding mosaicism and the absence of an off-target effect. However, there is major problem with this report and an extensive critique of it has been published.[77] Basically, the issue boils down to the lack of positive proof that any correction of the mutated allele took place. The authors of the original report[76] argued that, without any correction half of the embryos would carry a mutant allele, and that, since they observed substantial increase of embryos with the wild type allele only, they concluded that some of those embryos must represented gene correction. However, there are many possible reasons why a mutant allele was not detected in injected embryos[77] and these were not excluded in the original study. Actually, only the sequencing of the corrected allele, showing the presence of the original sequence followed by the inserted sequence (either from ssODN or from wild-type allele), followed in turn by the original sequence would represent incontrovertible evidence for gene correction. It is surprising that this evidence is lacking in the original report.[76]

Gene engineering in human embryos is obviously in its infancy and many technical problems need to be resolved if we are to see it used in clinical practice. Considering these uncertainties, we cannot but ask if this difficult and controversial technique is ever going to be necessary and justified. Novel mutations cannot be detected, thus cannot be corrected using this technique. Heterozygous parents with known recessive mutations will produce one quarter homozygous mutant embryos that can be corrected, thus avoiding the disease phenotype. Heterozygous parents with a dominant mutation will have one half of the progeny with the mutant phenotype which can also be avoided by gene correction. Nevertheless, gene correction in these situations is not essential since a much simpler, cheaper and more reliable approach, using preimplantation genetic diagnosis (PGD), can eliminate affected embryos. Some authors argued that by using gene correction there will be more embryos suitable for transfer thus increasing the chance of reproductive success.[76] This may be true, however, as we cannot distinguish which embryos need correction, we will have to target all of them with the potential for introducing off-target effects in otherwise completely normal embryos. For parents who insist on having their own genetic children there may be a situation in which gene correction in the zygote is the only possibility of having normal progeny. These are couples in which both parents are homozygous for a recessive mutation (e.g. β-thalassemia) or one or both parents are homozygous for a dominant mutation (e.g. Huntington disease). Such cases are rather rare and even for them one could envision a safer method for producing normal progeny. For example, the production of functional germ cells, derived from pluripotent stem cells, is approaching reality,[78,79] thus one could imagine deriving induced pluripotent cells from one or both parents, correcting the mutation and, following extensive study for off-target effects, deriving germ cells to be used in IVF.

In summary, genetic engineering in embryo is a fascinating possibility with many postulated uses, though it is hard to imagine its significant, massive application in human medicine.

Price Tag

The new clinical and therapeutic modalities described here also create new social and ethical problems. The cost of some clinical interventions like organ transplants or life-long maintenance therapy for certain genetic diseases is currently in the million dollar range as are

drug therapies for selected malignancies.[80,81] Are gene and stem cell therapies going to follow similar trends? [80,82] The first FDA-approved gene therapy for biallelic RPE65 mutation-associated retinal dystrophy, LUXTURNA, developed by Spark Therapeutic Inc. costs $ 850,000 per treatment.[83] One can easily argue that this price tag, for a quality of life improvement for a child who will now see, instead of being blind for life, is justified and even makes economic sense. However, at the present time we have only vague notions about the long-term success rate of these novel therapies, or how much will they cost, and who should finally pay for them. The cost of development and FDA-approval of treatment for very rare genetic diseases is in the billion dollar range and this cannot be recovered by charging the extremely small target population. It is very likely that stem cell therapies and gene engineering, the ultimate in personalized medicine, requiring highly skilled teams of molecular, cell and developmental biologists and clinicians will be even more expensive and will be available only at a very few specialized clinical centers. This brings us to the issue of access and justice[84] namely, will most of these therapies be available to the very rich only? How much is society willing to pay and what is the right cost-benefit ratio[85] for treatment? Is it fair to continue research funded by taxes, paid by all, if the products of this research will be available to only a few? At this moment most of these therapies are in the state of fascinating promises with many obvious biological and technical problems to be solved. One can only hope that by the time these problems are solved we will also have a clear notion of how to deal with the social and ethical conundrums arising.

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