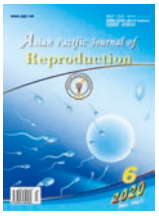

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# Overexpression of tyrosine phosphorylated proteins in reproductive tissues of polycystic ovary syndrome rats induced by letrozole

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## ABSTRACT

**Objective:** To identify the alteration of tyrosine phosphorylated protein expression in rats with polycystic ovary syndrome (PCOS).

**Methods:** Sixteen female Sprague-Dawley rats were divided into the control and letrozole-induced PCOS groups. The oestrus cycle of rats was performed by vaginal smear. Sex hormones and morphology of the ovary, oviduct, and uterus were observed. Expressions and intensity of androgen receptor and tyrosine phosphorylated proteins of reproductive organs were investigated by Western blot.

**Results:** Various polycysts and increased androgen receptor expression were present in the ovary of the PCOS group. The levels of follicle-stimulating hormone and testosteone were significantly higher in the PCOS group while progesterone and estradiol levels were significantly decreased as compared with the control group ($P<0.05$). Only the size of uterus in the PCOS group was significantly smaller than the control group. However, the density of collagen fibers observed in PCOS uterus was greater than the control group. Moreover, tyrosine phosphorylated proteins were significantly overexpressed in ovary (52, 42, and 28 kDa), oviduct (72, 56, 42, and 28 kDa), and uterus (53 and 42 kDa) of the PCOS group compared to the control group.

**Conclusions:** Presence of tyrosine phosphorylated proteins in the ovary, oviduct and uterus suggests that overexpression of tyrosine phosphorylated proteins may be involved in potential mechanism of female infertility especially in PCOS.

**KEYWORDS:** Polycystic ovary syndrome; Overexpression; Tyrosine phosphorylated proteins; Ovary; Oviduct; Uterus

## 1. Introduction

Female infertility is the most common problem affecting women reproductive ages around the world[1]. A major physiological cause of female infertility is anovulation or abnormal ovulation which is still clearly unexplained for its actual mechanism. It is well known that polycystic ovary syndrome (PCOS), a hypothalamic-pituitary-ovarian axis dysfunction, affects normal physiological ovulation[2]. In addition, the features of PCOS can be characterized by anovulation, hyperandrogenism, and polycystic ovaries[2]. Many factors can cause PCOS including vitamin D deficiency also assumed to related with insulin resistance[3]. Indeed, ovarian volume of PCOS woman is associated with serum luteinizing hormone level and luteinizing hormone/follicle-stimulating hormone ratio[4]. Additionally, it has been reported that the size of PCOS ovary is totally different between right and left sizes[5]. Unexpected anovulation of this condition is explained that PCOS can inhibit further ovarian follicular developments[6]. Then, undeveloped antral follicles will transform themselves to be follicular cysts. As a result, their granulosa cells turn to overproduce the androgen hormones especially testosterone[7]. Previously, Salley *et al*[8] reported that women with hyperandrogenism were associated with metabolic abnormalities. Moreover, when androgen gets higher than normal levels, the females will have unwanted symptoms in response to male sex hormones such as acne, alopecia, and hirsutism[9-11]. As already known, the excess androgens can also inhibit antral follicle

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development to be mature follicle, which causes anovulatory and irregular menstruation[9]. However, the specific pathogenesis of PCOS needs to be further explored.

Tyrosine phosphorylation is a post-transcriptional process playing essential roles in the cell proliferation, division, growth, and differentiation[12]. Basically, this process is required for sperm capacitation and acrosome reaction in female reproductive tracts before fertilization. In fertile male, the tyrosine phosphorylated proteins have been localized and identified in sertoli cells, spermatogenic cells, Leydig cells, epididymal epithelium, and seminal vesicle[13-15]. It has been demonstrated that tyrosine phosphorylated proteins were localized and identified in pig oocyte and assumed to be involved in the chromatin formation at metaphase[16]. Moreover, such proteins are also present in the uterine lysate of mice[17]. It has been shown that tyrosine phosphorylated protein profiles are totally different in each uterine cycle period of lizards[18]. Although many reports showed the changes of tyrosine phosphorylated protein expression in male reproductive tissues of infertile animal models[19-23], such issue in PCOS has never been documented. Therefore, this study attempted to investigate the alteration of tyrosine phosphorylated proteins in ovary, oviduct, and uterus of PCOS rat model.

## 2. Materials and methods

### 2.1. Animals and PCOS induction

Sixteen adult female Sprague–Dawley rats (6-8 weeks; weighing 160-200 g). Animals were housed in plastic cages with commercial pellet food and water ad libitum under the experiment room condition (12 h light/dark cycle, temperature (23±2) ℃, humidity 30%-60% relative humidity, sound < 85 decibels, and light intensity 350-400 lux) in Laboratory Animal Unit, Faculty of Medicine, Khon Kaen University, KhonKaen, Thailand. The rats were divided into the control and PCOS groups, with 8 rats in each group. The control group orally received 1% carboxy methyl cellulose solution, whereas the PCOS group orally received letrozole diluted in 1% carboxy methyl cellulose (1 mg/kg body weight) for consecutive 21 days. The vaginal smear was performed to confirm successful PCOS induction when abundant leukocytes were present on smeared slide. Then, the flushed vaginal lavage was smeared and stained with 0.1% crystal violet and further observed for specific cells to determine oestrous cycle.

### 2.2. Morphological studies

The gross morphology of the ovary, oviduct, and uterus was observed and captured by using a digital camera. Then, they were fixed with Bouin's solution for 48 h before routine paraffin-embedding. The paraffinized tissue blocks were sectioned at 5-7 μm (ERM 3100 Semi-Automatic Microtome, Hestion, Australia). All sections were stained with Masson's trichrome to study the pathology of collagen fibers. The histological images were taken by Nikon light microscope (Model: ECLIPSE E200, Japan) at 400× magnification

### 2.3. Western blotting

The total proteins of ovary, oviduct, and uterus were separated on 10% sodium dodecyl sulphate polyacrylamide gel by electrophoresis and transferred onto the nitrocellulose membrane. Subsequently, the transferred protein membranes were incubated with 5% skim milk to block non-specific binding proteins. All membranes were incubated with mouse anti-phosphotyrosine (Clone 4G10®, 1 : 2 000; Merck Millipore Co., USA) at 4 ℃ overnight followed by washing and incubating with mouse secondary antibody conjugated with horseradish peroxidase (1 : 2 000 dilution; Merck Millipore Co., USA) in 0.1% Tris-buffered saline for 1 h at room temperature. Epidermal growth factor lysate was used as positive control because the tyrosine amino acid residue of each factor was phosphorylated while the bovine serum albumin (non-phosphorylated) was used as negative control for tyrosine phosphorylated proteins detection. Additionally, the membrane of ovarian tissue was incubated with anti-androgen receptor (1 : 2 000 dilution) to confirm the increased-androgen expression, a marker for PCOS status. Then, all membranes were incubated with secondary antibody (anti-mouse) (1 : 2 000 dilution) in 0.1% TBST for 1 h at room temperature. To detect the target proteins (androgen receptor and tyrosine phosphorylated proteins), the enhanced chemiluminescence substrate reagent kit was used before visualization under gel documentation 4.

### 2.4. Relative intensity analysis

For quantitative analysis, the relative intensity of protein expression was measured and analysed by the ImageJ program (version 1.49) downloaded with 64-bit Java 1.8.0_172 (NIH, USA). The intensity of each protein band was quantified by using boundary setting of interest protein band with a rectangle frame to cover the minimum area containing the largest band of the row. The background with the same frame of one row of interested protein was taken into account. The signal intensity of protein bands in arbitrary units after normalization with the signal intensity of beta actin internal control.

### 2.5. Ethics statement

This study was approved by Animal Ethics Committee Research of

Faculty of Medicine Khon Kaen University, based on the Ethics of Animal Experimentation of National Research Council of Thailand (Record No. AEMDKKU 011/2019).

### 2.6. Serum hormonal assays

After collection of the blood from the cardiac puncture, blood was centrifuged at 9 000 ×*g* to separate the serum from the blood cells. Then the serum of each group was subjected into Roche/Hitachi Cobas C501 system (cobas® 6000 modular analyzer series, Roche Diagnostics Corporation) at the Diagnosis Clinical Chemistry Laboratory, Khon Kaen University Hospital, using ELISA kits for determination of the levels of luteinizing hormone, testosterone, progesterone and estradiol, respectively.

### 2.7. Statistical analysis

To compare the difference between two groups, the independent *t*-test was used for data normally- distributed or the Mann-Whitney *U* test was used for not normally-distributed data by using the program IBM SPSS statistics 19.0 software data analysis. This program was used to examine the significant difference between two groups. The *P* value < 0.05 was considered as a significant difference.

## 3. Results

### 3.1. Morphology and histology of female reproductive organs

In the gross structure comparison, the sizes of ovary and oviduct were not different between groups. Obviously, the size of uterus in the PCOS group was smaller than that of the control group (Figure 1A).

For histological study (Figure 1B), the ovary of the control rats showed normal developing follicles and corpus luteum (Figure 1Ba). In contrast, PCOS ovary showed various cystic follicles (Figure 1Bb, Bc). And no oocyte was observed within the cystic follicles (Figure Bc).

In addition, the histology of oviduct stained by Masson's trichrome was normal in both groups (Figure 2A). It was found that the intensity of collagen fibers within lamina propria and oviductal wall was not obviously different between the two groups (Figure 2A) Interestingly, the dimeter of uterus in the PCOS group seemed to be smaller as compared with that of the control group (Figure 2B), which corroborated its gross structure and weight shown in Figure 1. Moreover, the density of collagen fibers in PCOS uterus was obviously greater than that of the control uterus (Figure 2B).

### 3.2. Expression of androgen receptor in ovary

Expression of androgen receptor in ovary of the control and PCOS rats was demonstrated in the Figure 3 by using the testicular lystae as the possitive control for androgen receptor and equal beta actin expression as internal protein control. As expected, the results showed that the ovary of PCOS revealed the overexpression of the androgen receptor as compared to that of the control ovary (Figure 3).

### 3.3. Levels of serum hormones and the weight of reproductive organs

All serum sex hormome levels including luteinizing hormone, testosterone, progesterone, and estradiol were represented in the Table 1. The serum luteinizing hormone concentration and testosterone level in the PCOS group were significantly higher than those of the control group ($P<0.05$). Moreover, the serum levels of progesterone and estradiol were significantly decreased in the PCOS group as compared to the control group ($P<0.05$) (Table 1).

In addition, there was a significant increase in the body weight of the PCOS group as compared to the control group ($P<0.05$). Moreover, the absolute weight of ovary in the PCOS group was significantly increased, whereas the absolute weight of uterine in the PCOS group was significantly decreased as compared to the control group ($P<0.05$). The relative weight of uterus was also significantly decreased in the PCOS group as compared with the control group ($P<0.05$) (Table 2).

### 3.4. Effect of PCOS on expression of tyrosine phosphorylated proteins in female reproductive organs

The sodium dodecyl sulfate polyacrylamide gel electrophoresis stained by Coomassie blue demonstrated that the ovary (Figure 4A), oviduct (Figure 5A) and uterus (Figure 6A) of the control and PCOS rats showed similar protein profiles. The results showed four major tyrosine phosphorylated proteins (68, 52, 42, and 28 kDa) of ovary lysates in the control and PCOS rats (Figure 4B). However, the intensities of those proteins in PCOS ovary were significantly higher than those of the control group (Figure 4C).

In addition, the expressions of tyrosine phosphorylated proteins in oviduct lysates included 72, 56, 42, and 28 kDa in the control and PCOS rats (Figure 5B). However, the expressions of those tyrosine phosphorylated proteins in PCOS rats were significantly higher than the control group (Figure 5C).

Moreover, the results indicated that uterine protein profiles in both groups were similar and the tyrosine phosphorylated proteins were also found to be two major bands of 53 and 42 kDas (Figure 6B). Furthermore, the intensities of all tyrosine phosphorylated proteins were significantly increased in the PCOS group as compared with the control group ($P<0.05$) (Figure 6C).

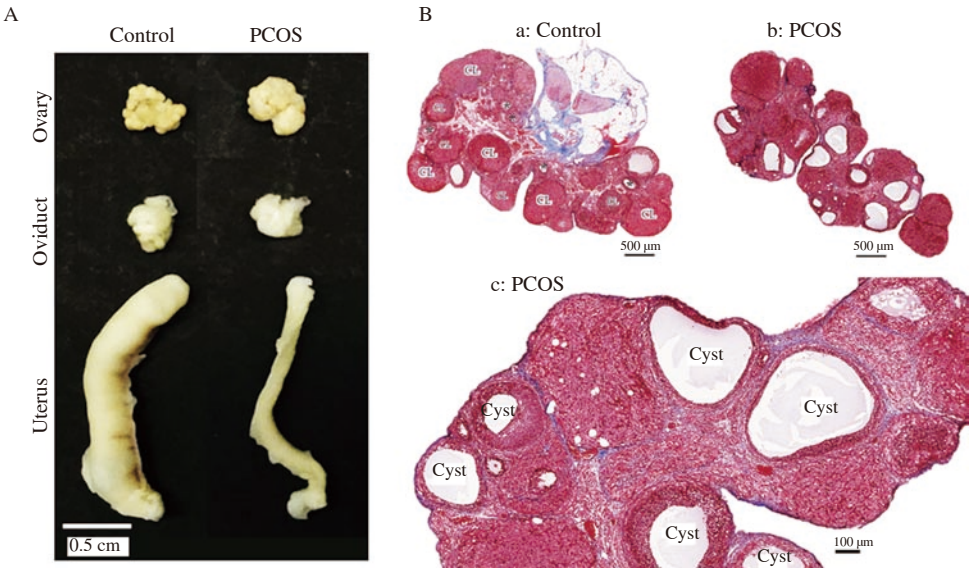


**Figure 1.** Representative gross morphological photograph of female reproductive organs including the ovary, oviduct and uterus (A), and histologies of ovary stained by Masson's trichrome of the control and PCOS rats (B). *: Normal follicles; CL: Corpus luteum; Cyst: cystic follicles.

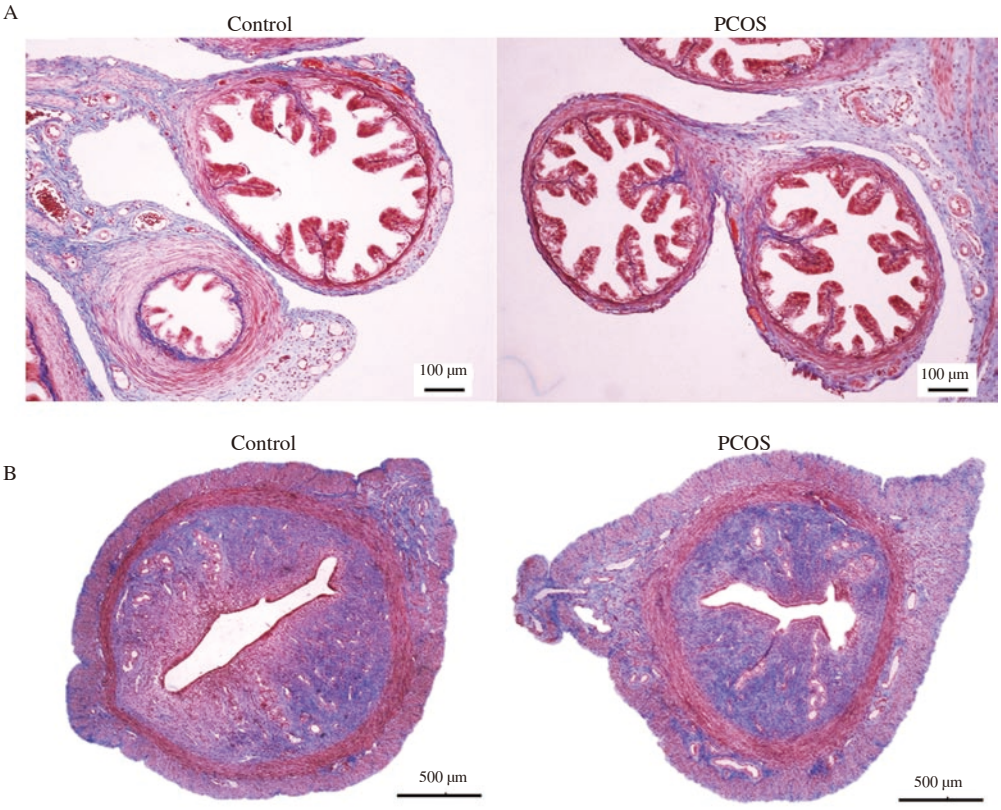


**Figure 2.** Representative histologies of oviduct (A) and uterus (B) stained by Masson's trichrome of the control and PCOS groups. There is no histopathology in epithelial cells lining on longitudinal folded lamina propria of oviduct in both groups. The diameter of the uterus in the PCOS group is smaller as compared with the control group. The density of collagen fibers in both oviducts is not different; however, its density in PCOS uterus is obviously greater than that of the control uterus.

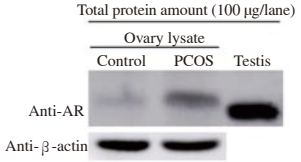


**Figure 3.** Expression of androgen receptor (AR) in the ovary of the control and PCOS rats. Testicular lysate is used as a positive control and β-actin is used as an internal control.

**Table 1.** Effects of PCOS on serum sex hormone levels as compared with the control female rats (mean±SD).

| Parameters | Control | PCOS | *P*-value |
|---|---|---|---|
| Luteinizing hormone (mIU/ mL) | 0.07±0.02 | 0.14±0.00 | 0.001 |
| Testosterone (ng/mL) | 1.81±0.01 | 7.80±0.09 | 0.001 |
| Progesterone (ng/mL) | 44.21±0.30 | 22.24±0.33 | 0.001 |
| Estradiol (pg/mL) | 78.01±0.01 | 23.66±0.30 | <0.001 |

PCOS: polycystic ovary syndrome.

**Table 2.** Comparison of increased body weight and female reproductive organ weights between the control and PCOS groups (mean±SD).

| Parameters | Control | PCOS | *P*-value |
|---|---|---|---|
| Increased body weight (%) | 18.49±1.70 | 43.71±3.44 | <0.001 |
| Ovary | | | |
| Absolute weight (g) | 0.06±0.01 | 0.08±0.01 | <0.001 |
| Relative weight (g/100 g body weight ) | 0.03±0.00 | 0.03±0.00 | 0.050 |
| Oviduct | | | |
| Absolute weight (g) | 0.013±0.010 | 0.009±0.003 | 0.050 |
| Relative weight (g/100 g body weight) | 0.002±0.000 | 0.004±0.001 | 0.050 |
| Uterus | | | |
| Absolute weight (g) | 0.240±0.100 | 0.070±0.004 | <0.001 |
| Relative weight (g/100 g body weight) | 0.130±0.050 | 0.030±0.002 | <0.001 |

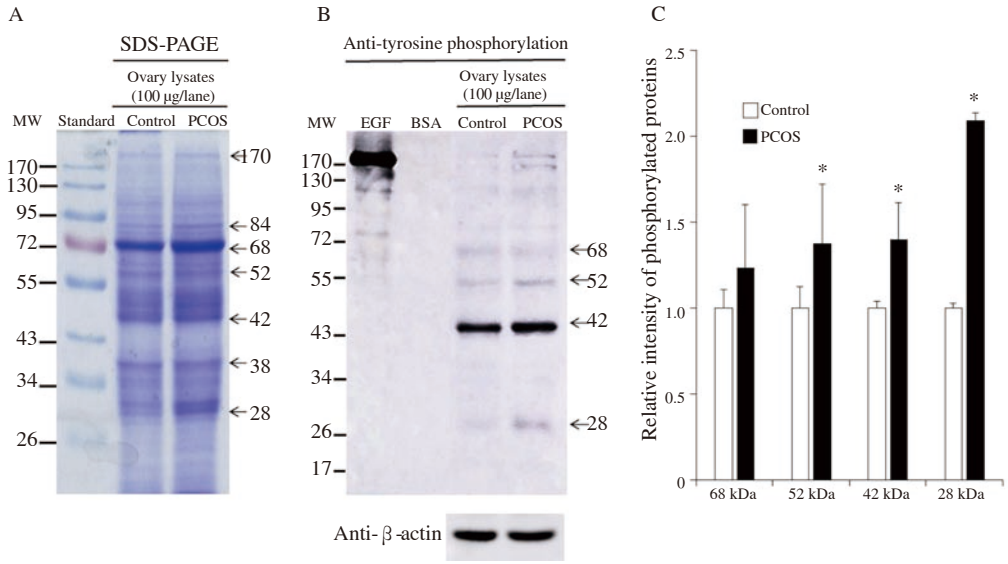


**Figure 4.** Tyrosine phosphorylation in the ovary lysates of the control and PCOS groups. A: Representative ovarian lysates protein profiles revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue; B: Western blotting of tyrosine phosphorylated proteins; C: Relative intensities of tyrosine phosphorylated proteins. *$P$<0.05: compared with the control group. BSA: bovine serum albumin used as a negative control for tyrosine phosphorylation; EGF: epidermal growth factor used as a positive control; kDa: kilodalton; MW: molecular weight. Standard: the ladder for reference.

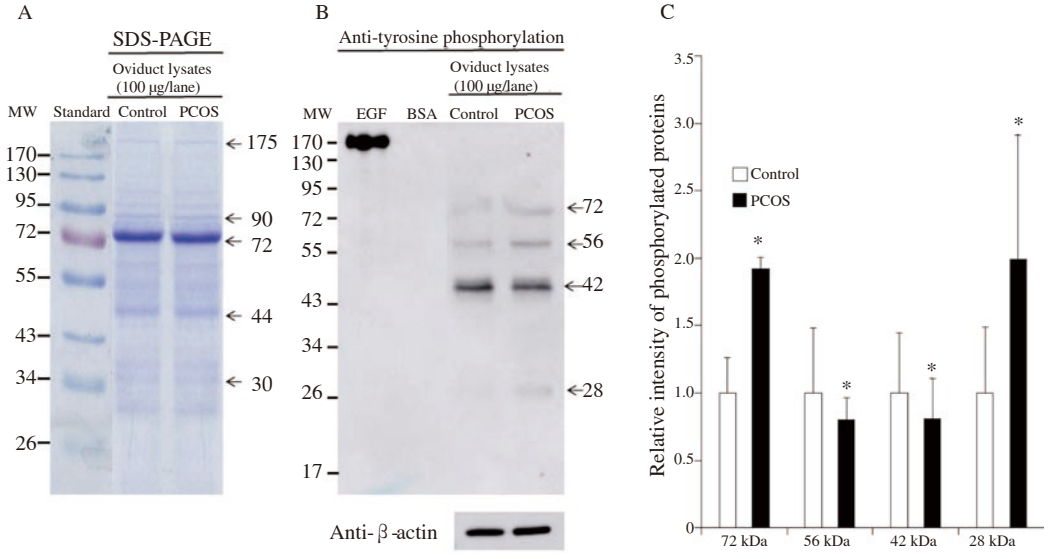


**Figure 5.** Tyrosine phosphorylation in the oviduct lysates of the control and PCOS groups. A: Representative oviduct protein profiles revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); B: Western blotting of tyrosine phosphorylated proteins in oviduct; C: Relative intensities of tyrosine phosphorylated proteins. *$P$<0.05: compared with the control group.

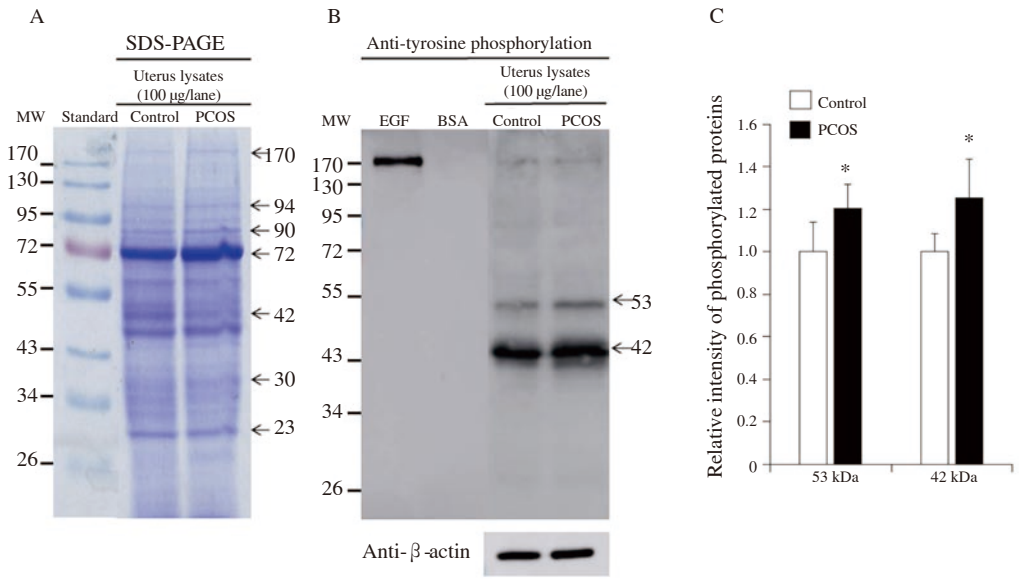


**Figure 6.** Tyrosine phosphorylation in the uterus lysates of the control and PCOS groups. A: Representative uterus protein profiles revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); B: Western blotting of tyrosine phosphorylated proteins in uterus; C: Relative intensities of tyrosine phosphorylated proteins. $^{*}P<0.05$: compared with the control group.

## 4. Discussion

Similar to human and animal models, many causes of PCOS may come from mental and psychological problems[25], lacking aromatase enzymatic activity in the granulosa cells in ovary[26], increase of oxidative stress[27], and *etc*. Such problems can cause the unexpected anovulation[28], resulting in female infertility[29]. In PCOS animal model, female rats used in this study adopting letrozole, a non-steroidal aromatase inhibitor for induction, has total PCOS features of vaginal cells, ovarian histology, and serum sex hormone as described in previous reports[30-33]. Moreover, this study further confirmed the biochemical PCOS condition by showing of the significantly increased expression of ovarian androgen receptor as compared to the control group. In consistence with other reports, our results also showed the polycystic ovary induced by letrozole as compared to control. Corroborated with their morphology and reduction of estradiol levels, the histology of oviduct and uterus of PCOS rats was obviously smaller than those of control.

Indeed, the significantly increased luteinizing hormone and testosterone levels in PCOS group indicated the actually physiological changes of PCOS condition confirming the successful induction. It is possible that the decreased progesterone and estradiol levels were associated with the decreased weights of oviduct and uterus. As the pathogenesis of PCOS is still being studied, we attempted to identify the changes of tyrosine phosphorylation in ovary including female reproductive tissues in comparison with those of the control group. It has been reviewed that proteomic samples from PCOS woman are totally different from healthy control women[34] by using proteomic techniques. Previous reports showed various additional proteins found in PCOS samples such as serum and plasma, ovarian tissue, and granulosa cells[35-38]. Our results have demonstrated the tyrosine phosphorylated proteins in POCS reproductive tissues. Moreover, it was found that the tyrosine phosphorylated proteins in ovary and reproductive tracts of PCOS were overexpressed. It indicates that some of such tyrosine phosphorylated proteins may be used as biomarkers for PCOS diagnosis. We assumed that a 68 kDa tyrosine phosphorylated protein found in PCOS ovary may be heat shock protein 70 (Hsp70) linked with tyrosine phosphate because it has been reported in contribution to PCOS pathology[39]. In oviduct of PCOS rats, it is possible that a 72 kDa tyrosine phosphorylated protein could also be Hsp70 which has been shown to be present in estrous and luteal phases of mammalian oviduct[40]. Additionally, it has been demonstrated that Hsp70 plays the important roles in pathologic conditions including PCOS. Indeed, Hsp70 has been shown to have post-translational modifications especially phosphorylation for its biological activity. Moreover, this study showed the same band of 42 kDa tyrosine phosphorylated protein in both oviduct and uterus of the control and PCOS rats, indicating the same roles in reproductive tracts. As previously reported, this study hypothesized that such protein may be an enzyme called isocitrate dehydrogenase 1 (about 42-46 kDas), responsible for cellular detoxification processes in response to oxidative stress. In addition, it was assumed that the possible tyrosine phosphorylated proteins which are overexpressed in PCOS ovary may be responsible for compensation to maintain normal ovarian functions such as oogenesis, ovulation, and steroidogenesis. A previous study reported that the developing ovarian follicles contain approximately 1 401 identified proteins by using proteomic tools[41]. It is possible that the ovarian tyrosine phosphorylated proteins observed in this study are included. Indeed, some tyrosine phosphorylated proteins have

been investigated in ovary and are involved in ovarian follicular development. Such possible ovarian proteins include the platelet-derived growth factors[42], nerve growth factor, angiogenic factors like vascular endothelial growth factor and transforming growth factor beta1, and transforming growth factors beta and alpha[43], respectively.

This paper has addressed some of the limitations about characterization of TyrPho proteins expressed in rat female reproductive tracts and ovary because total protein concentration extracted were not enough to perform immunopricipitaion and proteomic analyses.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

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## Authors' contributions

Sudtida Bunsueb contributed to animal administration, data collection, statistical analysis, and first draft manuscript preparation. Arada Chaiyamoon contributed to data collection and histological analyses. Supatcharee Arun contributed to critical discussion and result consultancy. Alexander Tsang-Hsien Wu contributed to critical discussion and proofreading. Sitthichai Iamsaard contributed to experimental design, critical discussion, and developing the manuscript.

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