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Changes of testicular phosphorylated proteins in response to restraint stress in male rats*

Supatcharee ARUN[1], Jaturon BURAWAT[1], Wannisa SUKHORUM[1], Apichakan SAMPANNANG[1], Nongnut UABUNDIT[1], Sitthichai IAMSAARD[†‡1,2]

([1]*Department of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*)

([2]*Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*)

[†]E-mail: sittia@kku.ac.th

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Abstract: Objective: To investigate male reproductive parameters via changes of potential testicular protein markers in restraint-stress rats. Methods: Male Sprague-Dawley rats were divided into two groups (non-immobilized control and restraint-immobilized/stress groups, n=8 each group). The stress animals were immobilized (12 h/d) by a restraint cage for 7 consecutive days. All reproductive parameters, morphology and histology were observed and compared between groups. In addition, the expression of steroidogenic acute regulatory (StAR) and phosphotyrosine proteins (previously localized in Sertoli and late spermatid cells) in testicular lysate was assayed by immuno-Western blotting. Results: Testosterone level, sperm concentration and sperm head normality of stress rats were significantly decreased while the corticosterone level was increased as compared with the control (P<0.05). Histologically, stress rats showed low sperm mass in epididymal lumen and some atrophy of seminiferous tubules. Although the expression of testicular StAR protein was not significantly different between groups, changed patterns of the 131, 95, and 75 kDa testicular phosphorylated proteins were observed in the stress group compared with the control group. The intensity of a testicular 95-kDa phosphorylated protein was significantly decreased in stress rats. Conclusions: This study has demonstrated the alteration of testicular phosphorylated protein patterns, associated with adverse male reproductive parameters in stress rats. It could be an explanation of some infertility in stress males.

Key words: Restraint-stress rats, Steroidogenic acute regulatory (StAR) protein, Testicular phosphorylated protein
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1 Introduction

Stress has come to the fore as a major factor adversely affecting the quality of human life. It affects various physiological processes including reproductive functions. Numerous studies in human and experimental animals have shown that stress causes adverse effects in the male reproductive system: (1) erectile dysfunction (Nathan, 1986; Ernst *et al*., 1993; Kennedy *et al*., 1999), (2) decrease of sperm quality (Almeida *et al*., 1998; Clarke *et al*., 1999; Hari Priya and Sreenivasula Reddy, 2012; Hari Priya *et al*., 2014; Rao *et al*., 2015; Zhang *et al*., 2015), (3) decrease of testosterone levels (Orr and Mann, 1990; Retana-Márquez *et al*., 2003; Weissman *et al*., 2009; Lin *et al*., 2014; Prabsattroo *et al*., 2015), and (4) damage to testicular tissue (Rai *et al*., 2003; 2004; Aziz *et al*., 2013; Prabsattroo *et al*., 2015). Indeed, the corticosterone levels are markedly elevated under a restraint immobilization condition (Bhatia *et al*., 2011; Prabsattroo *et al*., 2015). Corroborated with the decrease of testosterone levels, Lin *et al*. (2014) demonstrated that stress could decrease the expression

‡ Corresponding author

* Project supported by the Postgraduate Study Support Grant and Invitation Research Grant (IN59134), Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

ORCID: Supatcharee ARUN, http://orcid.org/0000-0002-7518-2406; Sitthichai IAMSAARD, http://orcid.org/0000-0002-6793-2879

of steroidogenic acute regulatory (StAR) protein and the cytochrome P450 side chain cleavage enzyme (CYP11A1) in rat Leydig cells after acute immobilization stress induction.

Protein tyrosine phosphorylation is a post-transcriptional process that is important for the regulation and coordination of various cell proliferation, division, growth, and differentiation function in both normal and cancer cells (Hunter and Cooper, 1985; Hunter, 1987; Hanks *et al*., 1988; Ullrich and Schlessinger, 1990). In testicular tissue, the phosphorylated proteins have been localized in the Sertoli cells and late (elongated) spermatids (except in the Leydig cells), and these proteins are assumed to have the roles in spermatogenesis (Arad-Dann *et al*., 1993). In addition, sperm capacitation and acrosome reaction in the fertilization steps require protein tyrosine phosphorylation (Kopf and Gerton, 1991; Yanagimachi, 1994; Visconti and Kopf, 1998). Although it has been shown that some drugs or substances can change the expression of testicular phosphorylated proteins (Ballester *et al*., 2004; Iamsaard *et al*., 2013; 2014), these changes in stress events have never been reported. This study, therefore, attempted to demonstrate the alterations of testicular phosphorylation in stress rats.

2 Materials and methods

2.1 Animals and stress procedure

Male Sprague-Dawley rats (200–250 g) were purchased from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The rats were administered commercially available pellet and water ad libitum in plastic cages under controlled environmental conditions (temperature (22±2) °C; 12 h light/dark cycles). This study was approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of National Research Council of Thailand (Ref. No. AEKKU-NLAC 11/2558). After a week of acclimatization, animals were randomly divided into two groups (n=8). Group 1 (control group) was not immobilized by a restraint cage and Group 2 (restraint-stress group) was immobilized by a restraint cage (12 h/d to induce acute stress; as described by Ahmad *et al*. (2012), Retana-Márquez *et al*. (2003), and Prabsattroo *et al*. (2015)), followed by weighing the body for 7 consecutive days.

2.2 Plasma corticosterone and testosterone assays

After animals were euthanized, the blood was collected by cardiac puncture of the left ventricle and centrifuged at 13000 r/min at 4 °C for 7 min by Microfuge 22R (Biocompare Inc., USA) to separate the plasma serum from the blood cells. Subsequently, all blood sera were sent to the Radiology Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand, for measurement of serum corticosterone and testosterone levels.

2.3 Morphological and histological studies

At the end of the experiment, all rats were euthanized by cervical dislocation and sacrificed to collect male reproductive organs (testis, penis, epididymis plus vas deferens, and seminal vesicle). Subsequently, these organs were weighed after the fats were removed. The reproductive organ weights were calculated and expressed as the relative reproductive organ weights (g/100 g). Then, all organs were observed for their gross structures and their images captured by digital camera (Nikon Coolpix S2600, Japan). To examine their histology, the testis, caudal epididymis, and penis were fixed in 10% phosphate buffered formalin (pH 7.4) for 24 h and routinely processed for light microscope technique. All the sections of testis, caudal epididymis, and penis were stained with hematoxylin and eosin (H&E), whereas the penis sections were also stained with Masson's trichrome (Sigma-Aldrich Inc., USA) to investigate collagen fibers. Finally, histological photographs were taken by a Nikon light ECLIPSE E200 microscope (Nikon Inc., Japan) equipped with a DXM1200 digital camera (Nikon Inc., Japan) and an ImageJ program (Version 1.49p) was used to quantify the collagen fiber.

2.4 Sperm concentration and head morphology assessment

The left caudal epididymis and vas deferens were operated on gently and squeezed for sperm fluid. Epididymal sperm fluid was dipped and suspended in 1 ml of phosphate buffer saline (PBS; 37 °C, pH 7.4). Subsequently, the diluted sperm were centrifuged at 5000 r/min at 25 °C for 2 min to wash and separate mature sperm pellets from the fluid. The sperm pellets were re-suspended with 1 ml PBS (37 °C, pH 7.4). Then the sperm suspension was diluted with PBS (1:20 (v/v) dilution) before a count was made of the

mature sperm using a Neubauer counting chamber under a light microscope (Nikon ECLIPSE E200, Japan) in triplicate examinations (Iamsaard *et al*., 2013). To examine sperm head abnormalities, described by Wyrobek and Bruce (1975) and Sakr *et al*. (2014), the diluted sperm (10 μl) were smeared onto a cleaned glass slide. The smears were air-dried and incubated in a hot air oven at 50 °C overnight. The dried sperm were fixed in methyl alcohol and stained by H&E. Abnormal sperm heads were classified and explained by Sakr *et al*. (2014). All sperm heads were captured by a Nikon light ECLIPSE E200 microscope equipped with a DXM1200 digital camera. To quantify sperm head abnormalities, a total of 600 sperms were counted in each animal. Then the abnormalities were calculated and represented as a percentage of sperm head abnormality.

2.5 Western blot analysis of StAR and phosphotyrosine protein expression

Testicular tissue was homogenized with radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology Inc., USA) containing a cocktail of protease inhibitors (Sigma Inc., USA) to extract and maintain the proteins. Then the testicular homogenate was centrifuged at 12000 r/min at 4 °C for 10 min to separate testicular lysate from pellet. The total protein concentrations of the testicular lysate were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA) at an absorbance of 280 nm. To determine testicular StAR protein expression, the total testicular proteins (80 μg) of both groups were loaded and separated on 10% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose membrane. Subsequently the membrane was incubated with 5% skim milk in 0.1% PBST (0.1% Tween-20, 0.01 mol/L PBS, pH 7.4) for 1 h to block non-specific binding proteins and then incubated with StAR antibody (1:1000 (v/v) dilution; Santa Cruz Biotechnology Inc., USA) or β-actin antibody (1:2000 (v/v) dilution; Santa Cruz Biotechnology Inc., USA) at 4 °C overnight. The membrane was washed in 0.05% PBST (0.05% Tween-20, 0.01 mol/L PBS, pH 7.4) for 5 min (three times) and incubated with goat anti-rabbit IgG or goat anti-mouse conjugated horseradish peroxidase (HRP) secondary antibody for 1 h at room temperature. For the analysis of testicular phosphotyrosine protein expression as described by Iamsaard *et al*. (2003; 2004), the transferred-protein membrane was incubated with anti-phosphotyrosine primary antibody (1:2000 (v/v); Millipore Co., USA) at 4 °C overnight followed by washing and then incubated with anti-mouse conjugated with HRP secondary antibody for 2 h at room temperature. Then, all membranes were washed in 0.05% PBST before detection of StAR or β-actin using enhanced chemiluminescence (ECL) substrate under gel doct 4 (ImageQuant 400, GH Healthcare, USA). For protein expression, the ImageJ program (Version 1.49p) was used to analyze the relative intensity of target proteins.

2.6 Statistical analysis

All quantitative data were represented as mean± standard deviation (SD). The independent sample *t*-test was performed to examine the significant difference between two groups using SPSS statistics 19.0 software. A *P*-value of <0.05 was considered as a significant difference.

3 Results

3.1 Effect of stress on rat body weights and male reproductive organs

Daily changes of the body weight in the control and restraint-stress groups are shown in Fig. 1a. This result demonstrated that the body weights of stress group were significantly decreased ($P<0.05$) in consecutive 7 d as compared with the control group. Effect of stress on morphology of male reproductive organs was also investigated. The results showed that the testis of restraint-stress group was smaller than that of the control group (Fig. 1b). The testicular size (width× length) was (1.31±0.02) cm×(2.26±0.02) cm for the control and (1.25±0.01) cm×(2.08±0.01) cm for the stress group, respectively. However, morphologies of the penis, epididymis plus vas deferens, and seminal vesicles in both groups were not significantly different (Fig. 1b).

3.2 Effect of stress on sperm head morphology

The sperm head morphologies of the control and restraint-stress groups are shown in Fig. 2. Normal sperm heads were mostly observed in the control group (Fig. 2a). In contrast, abnormal sperm heads

(without the hook (Fig. 2b), pinhead (Fig. 2c), and crooked neck (Fig. 2d)) were increased in the restraint-stress group.

3.3 Effect of stress on the weights of reproductive organs, sperm concentration, sperm head abnormality, and corticosterone hormone

The absolute and relative weights of the testis, epididymis plus vas deferens, and seminal vesicle in the restraint-stress and control groups were shown in Table 1. Significantly, the absolute and relative weights of testes were reduced (P<0.05) in the restraint-stress group. However, the rest parameters were not significantly different between both groups. In contrast, the restraint stress significantly decreased (P<0.05) sperm concentration and testosterone level while it significantly increased (P<0.05) the percentage of sperm head abnormality and corticosterone level compared with the control group (Table 1).

3.4 Effect of stress on histology of testis, caudal epididymis, and penis

The histology of male reproductive organs is shown in Fig. 3. The result showed that the stress moderately damaged seminiferous tubules by increasing tubular atrophy and interstitial space compared with the control group (Figs. 3a and 3b). In line

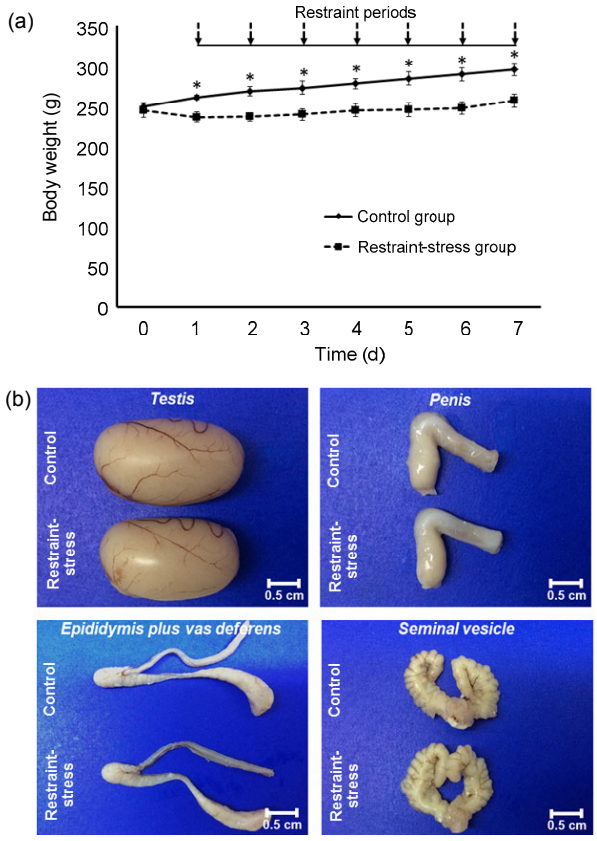

Fig. 1 Body weights (a) and comparative morphologies of testis, penis, epididymis plus vas deferens, and seminal vesicle (b) between the control and restraint-stress groups
Each data point in (a) is represented as mean±SD (n=8 rats each group). * P<0.05, vs. restraint-stress group

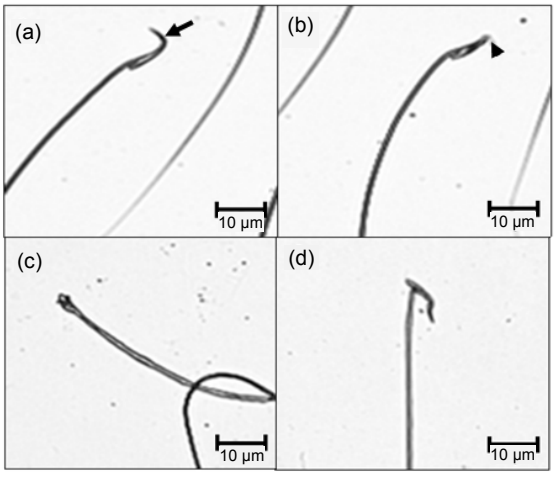

Fig. 2 Photographs showing sperm head morphologies observed in the control and restraint-stress groups
Normal sperm head with normal hook (arrow) (a); abnormal sperm heads without hook (arrow head) (b), pinhead (c), and crooked neck (d)

Table 1 Effect of stress on the weights of male reproductive organs, sperm concentration, sperm head abnormality, and corticosterone levels in rats

Group	Absolute weight (g)			Relative weight (g/100 g)			c_s (10^6 cells/ml)	Sperm head abnormality (%)	c_c (ng/ml)	c_t (ng/ml)
	Testis	Epididymis plus vas deferens	Seminal vesicle	Testis	Epididymis plus vas deferens	Seminal vesicle				
Control	1.60±0.09	0.40±0.01	0.64±0.04	0.54±0.04	0.14±0.01	0.22±0.01	167.00±2.53	1.38±0.86	389.00±23.00	1.08±0.46
RS	1.26±0.15*	0.38±0.01	0.63±0.07	0.49±0.06*	0.15±0.00	0.25±0.03	69.50±8.98*	2.75±0.66*	506.20±57.47*	0.47±0.26*

RS: restraint-stress; c_s: sperm concentration; c_c: corticosterone level; c_t: testosterone level. * Significant differences (P<0.05), compared with the control group. Data are represented as mean±SD (n=8 rats each group)

with sperm concentration (Table 1), the density of caudal sperm in the restraint-stress group was markedly lower than that in the control group (Figs. 3c and 3d). However, the histology of penis and penile collagen fibers stained by H&E and Masson's trichrome was not different between the control and restraint groups (Figs. 3e–3h).

3.5 Effect of stress on testicular StAR protein

StAR protein expression was shown by immuno-Western blotting (Fig. 4a). The results showed that restraint stress did not affect testicular StAR protein expression compared with the control (Fig. 4a). As confirmed by the relative intensity of StAR protein and the ratio of StAR/β-actin (Figs. 4b and 4c), the results showed that StAR protein levels were not significantly different in both groups.

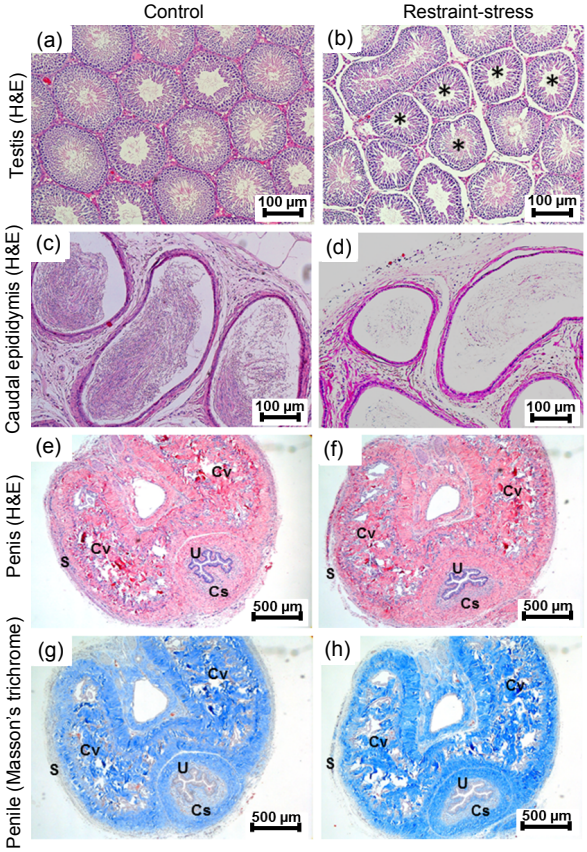

Fig. 3 Photomicrographs showing histologies of testis (a, b), caudal epididymis (c, d), and penis (e, f) stained by H&E and penile section stained by Masson's trichrome (g, h) of the control and restraint-stress groups, respectively
Asterisks: atrophic seminiferous tubules; S: sheath of tunica albugenia; Cv: corpora cavernosa; Cs: corpus spinosum; U: urethra

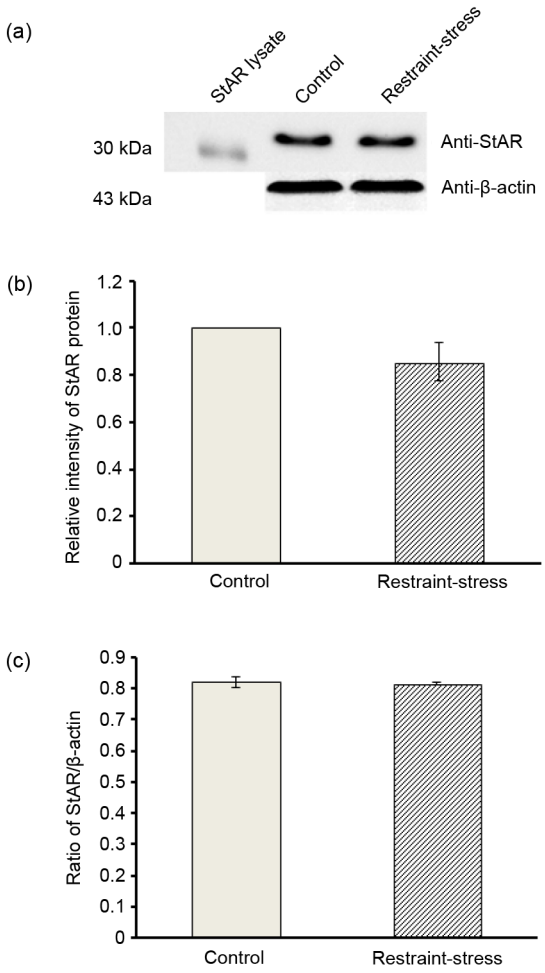

Fig. 4 Representative Western-blot analysis of StAR protein expression in testicular lysates (a), relative intensity of testicular StAR protein (b), and ratio of StAR/β-actin (c) in the control and restraint-stress groups
StAR lysate was used as a positive control. Data are represented as mean±SD (n=4 rats each group)

3.6 Effect of stress on testicular tyrosine protein phosphorylation

The effect of stress on testicular phosphotyrosine expression is shown in Fig. 5a. The result showed that six testicular phosphorylated protein bands (48, 65, 75, 95, 131, and 200 kDa) were clearly detected in both control and restraint-stress groups (Fig. 5a). Intriguingly, we found the decreased expression of a phosphorylated 95-kDa protein in the restraint-stress group with normal protein profiles by SDS-PAGE compared with control (Fig. 5b). This result was confirmed by its relative intensity (Fig. 5c).

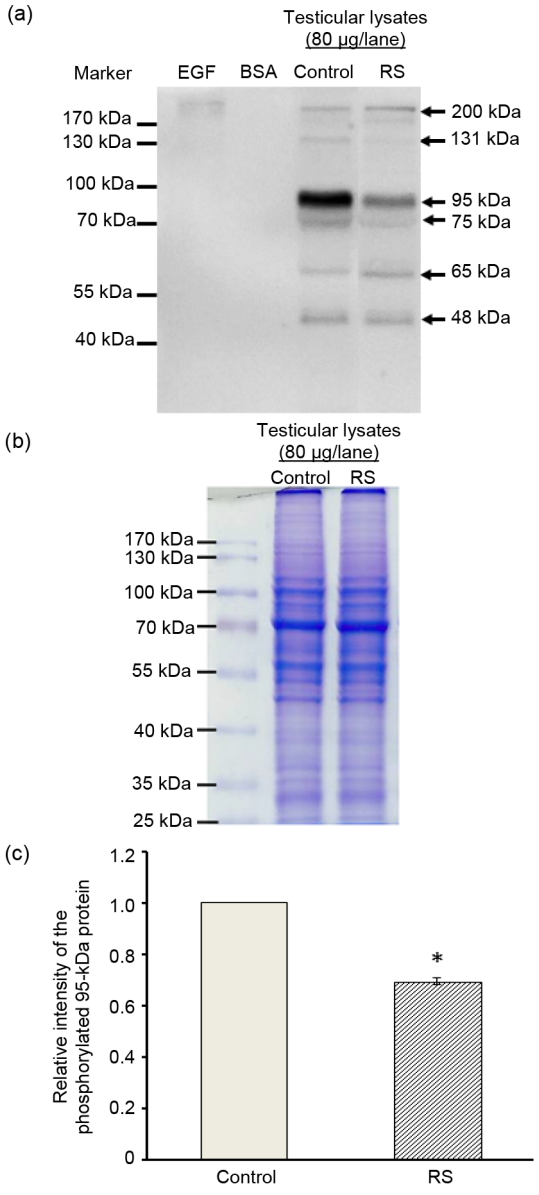

Fig. 5 Representative Western-blot analysis of tyrosine protein phosphorylation in testicular lysates (a), protein profiles (SDS-PAGE) (b), and relative intensity of the testicular phosphorylated 95-kDa protein (c) in the control and restraint-stress (RS) groups

Bovine serum albumin (BSA) and epidermal growth factor (EGF)-like growth factors were used as negative and positive controls, respectively. Data were represented as mean±SD (n=4 rats each group). * P<0.05, compared with the control group

4 Discussion

Stress is a major factor in male infertility. It has been reported as inducing erectile dysfunction, premature ejaculation, orgasmic difficulty, and low sperm quality (Nathan, 1986; Ernst *et al*., 1993; Clarke *et al*., 1999; Kennedy *et al*., 1999; Rao *et al*., 2015; Zhang *et al*., 2015). In the recent study, all parameters of adverse male reproductive system including decreased body weight in rats exposed to restraint stress (Fig. 1a and Table 1) were similar to those parameters previously documented (Orr and Mann, 1990; Carrasco and van de Kar, 2003; Zardooz *et al*., 2006; Weissman *et al*., 2009; Hari Priya and Sreenivasula Reddy, 2012; Lin *et al*., 2014; Prabsattroo *et al*., 2015). In explanation of weight loss (Table 1), it is known that testosterone activates the protein synthetic apparatus muscles and other organs while corticosterone increases protein catabolism and decreases protein synthesis. In addition, many previous studies showed that increased corticosterone mediated restraint-induced weight loss via increasing glycolysis and lipolysis (Arner, 1992; Lafontan *et al*., 1997; Chotiwat and Harris, 2008; Scherer *et al*., 2011). Consistent with previous studies (Hari Priya and Sreenivasula Reddy, 2012; Bitgul *et al*., 2013), this study also demonstrated atrophy of seminiferous tubules in stress testes (Fig. 3b). In addition, the low density of epididymal sperm mass was also shown in stress rats (Fig. 3d), and it was associated with a significant decrease of sperm concentration (Table 1). Interestingly, we found that sperm head abnormality of stress rats was significantly greater than that of control (Table 1 and Fig. 2). Although restraint stress can suppress the activities of monoamine oxidase type B and phosphodiesterase type 5 in the rat penis (Prabsattroo *et al*., 2015), it affects neither penile morphology nor the amount of collagen fibers (Figs. 1b and 3e–3h). This result indicated that stress interrupted penile function but not its morphology.

This study attempted to explain the decrease of testosterone levels in stress rats by observing StAR protein expression using Western bolt (Fig. 4). Unexpectedly, the expression of StAR levels in both groups was not significantly different (Figs. 4a–4c). It is possible that StAR expression is very sensitive to acute immobilization for a day (Lin *et al*., 2014). In the subacute stress for consecutive 7 d (present study), this expression might be already corrected to be at normal levels (Fig. 4). To additionally clarify the decreased testosterone levels, the disturbances of other steroidogenic machineries such as cytochrome P-450 cholesterol side-chain cleavage enzymes,

scavenger receptor class B, and hydroxysteroid dehydrogenases need to be further investigated. In various studies, the increase of malondialdehyde levels and the decrease of enzymatic activities of catalase, glutathione, and superoxide dismutase have been demonstrated in stress testicular tissue (Zayachkivska *et al*., 2006; Brzozowski *et al*., 2008; Bhatia *et al*., 2011; Warzecha *et al*., 2011; Kwiecien *et al*., 2012; Hari Priya and Sreenivasula Reddy, 2012; Xu *et al*., 2014). The alteration of oxidative stress markers might affect normal spermatogenesis, resulting in decreased sperm concentration (Table 1 and Fig. 3d). When probed with an anti-phosphotyrosine monoclonal antibody, the phosphorylation of testicular proteins has been localized in only Sertoli and elongated spermatid cells (Arad-Dann *et al*., 1993). It indicates that testicular tyrosine-phosphorylated proteins play some roles in spermatogenesis since this post-translational process is involved in proliferation and differentiation (Hunter and Cooper, 1985; Hunter, 1987; Hanks *et al*., 1988; Ullrich and Schlessinger, 1990). Moreover, it is well documented that sperm phosphorylated proteins are essential for capacitation and acrosome reaction (Kopf and Gerton, 1991; Yanagimachi, 1994; Visconti and Kopf, 1998). The patterns of testicular phosphorylated proteins have also been previously shown in hyperglycemic rats (Ballester *et al*., 2004) and they are changed when treated with some drugs or substances, resulting in alterations of sperm concentration (Iamsaard *et al*., 2013; 2014). In the same vein, we have demonstrated for the first time that restraint stress could change the pattern of testicular phosphorylated protein of stress rats compared with the control healthy rats (Fig. 5). The relative intensity of a phosphorylated 95-kDa protein band in stress testicular lysate was significantly decreased (Figs. 5a–5c), which was associated with the decrease of sperm concentration and sperm head morphology (Table 1 and Fig. 2). Taken together, we speculate that this phosphorylated protein pattern might play an important role in spermatogenesis, especially in the differentiation process of spermatogenesis.

Compliance with ethics guidelines

Supatcharee ARUN, Jaturon BURAWAT, Wannisa SUKHORUM, Apichakan SAMPANNANG, Nongnut UABUNDIT, and Sitthichai IAMSAARD declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

References

Ahmad, A., Rasheed, N., Gupta, P., *et al*., 2012. Novel Ocimumoside A and B as anti-stress agents: modulation of brain monoamines and antioxidant systems in chronic unpredictable stress model in rats. *Phytomedicine*, **19**:639-647. http://dx.doi.org/10.1016/j.phymed.2012.02.012

Almeida, S.A., Petenusci, S.O., Anselmo-Franci, J.A., *et al*., 1998. Decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty. *Braz. J. Med. Biol. Res*., **31**(11):1443-1448. http://dx.doi.org/10.1590/S0100-879X1998001100013

Arad-Dann, H., Beller, U., Haimovitch, R., *et al*., 1993. Immunohistochemistry of phosphotyrosine residues: identification of distinct intracellular patterns in epithelial and steroidogenic tissues. *J. Histochem. Cytochem*., **41**(4): 513-519. http://dx.doi.org/10.1177/41.4.7680679

Arner, P., 1992. Adrenergic receptor function in fat cells. *Am. J. Clin. Nutr*., **55**:228S-236S.

Aziz, N.M., Ragy, M.M., Gayyed, M.F., 2013. Effect of acute immobilization stress with or without a heme oxygenase inducer on testicular structure and function in male albino rats. *J. Basic Clin. Physiol. Pharmacol*., **24**(4):255-262. http://dx.doi.org/10.1515/jbcpp-2012-0066

Ballester, J., Munoz, M.C., Dominguez, J., *et al*., 2004. Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *J. Androl*., **25**(5):706-719. http://dx.doi.org/10.1002/j.1939-4640.2004.tb02845.x

Bhatia, N., Jaggi, A.S., Singh, N., *et al*., 2011. Adaptogenic potential of curcumin in experimental chronic stress and chronic unpredictable stress-induced memory deficits and alterations in functional homeostasis. *J. Nat. Med.*, **65**(3-4):532-543. http://dx.doi.org/10.1007/s11418-011-0535-9

Bitgul, G., Tekmen, I., Keles, D., *et al*., 2013. Protective effects of resveratrol against chronic immobilization stress on testis. *Urology*, **6**:1-10. http://dx.doi.org/10.1155/2013/278720

Brzozowski, T., Konturek, P.C., Chlopicki, S., *et al*., 2008. Therapeutic potential of 1-methylnicotinamide against acute gastric lesions induced by stress: role of endogenous prostacyclin and sensory nerves. *J. Pharmacol. Exp. Ther*., **326**(1):105-116. http://dx.doi.org/10.1124/jpet.108.136457

Carrasco, G.A., van de Kar, L.D., 2003. Neuroendocrine pharmacology of stress. *Eur. J. Pharmacol*., **463**(1-3):235-272. http://dx.doi.org/10.1016/S0014-2999(03)01285-8

Chotiwat, C., Harris, R.B., 2008. Antagonism of specific corticotropin-releasing factor receptor subtypes selectively modifies weight loss in restrained rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **295**(6):R1762-R1773. http://dx.doi.org/10.1152/ajpregu.00196.2008

Clarke, R.N., Klock, S.C., Geoghegan, A., *et al*., 1999. Relationship between psychological stress and semen quality among in-vitro fertilization patients. *Hum. Reprod*., **14**(3): 753-758.

http://dx.doi.org/10.1093/humrep/14.3.753

Ernst, C., Foldenyi, M., Angst, J., 1993. The Zurich Study: XXI. Sexual dysfunctions and disturbances in young adults. Data of a longitudinal epidemiological study. *Eur. Arch. Psychiatry Clin. Neurosci*., **243**(3-4):179-188. http://dx.doi.org/10.1007/BF02190725

Hanks, S.K., Quinn, A.M., Hunter, T., 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**(4861):42-52. http://dx.doi.org/10.1126/science.3291115

Hari Priya, P., Sreenivasula Reddy, P., 2012. Effect of restraint stress on lead-induced male reproductive toxicity in rats. *J. Exp. Zool. A Ecol. Genet. Physiol*., **317**(7):455-465. http://dx.doi.org/10.1002/jez.1738

Hari Priya, P., Girish, B.P., Sreenivasula Reddy, P., 2014. Restraint stress exacerbates alcohol-induced reproductive toxicity in male rats. *Alcohol*, **48**(8):781-786. http://dx.doi.org/10.1016/j.alcohol.2014.07.014

Hunter, T., 1987. A thousand and one protein kinases. *Cell*, **50**(6):823-829. http://dx.doi.org/10.1016/0092-8674(87)90509-5

Hunter, T., Cooper, J.A., 1985. Protein tyrosine kinases. *Annu. Rev. Biochem*., **54**(1):897-930. http://dx.doi.org/10.1146/annurev.bi.54.070185.004341

Iamsaard, S., Prabsattroo, T., Sukhorum, W., *et al*., 2013. *Anethum graveolens* Linn. (dill) extract enhances the mounting frequency and level of testicular tyrosine protein phosphorylation in rats. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **14**(3):247-252. http://dx.doi.org/10.1631/jzus.B1200287

Iamsaard, S., Arun, S., Burawat, J., *et al*., 2014. Phenolic contents and antioxidant capacities of Thai-Makham Pom (*Phyllanthus emblica* L.) aqueous extracts. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **15**(4):405-408. http://dx.doi.org/10.1631/jzus.B1300284

Kennedy, S.H., Dickens, S.E., Eisfeld, B.S., *et al*., 1999. Sexual dysfunction before antidepressant therapy in major depression. *J. Affect. Disord*., **56**(2-3):201-208. http://dx.doi.org/10.1016/S0165-0327(99)00050-6

Kopf, G., Gerton, G., 1991. The mammalian sperm acrosome and the acrosome reaction. *In*: Wassarman, P. (Ed.), Elements of Mammalian Fertilization. CRC Press, Boca Raton, USA, p.154-203.

Kwiecien, S., Ptak-Belowska, A., Krzysiek-Maczka, G., *et al*., 2012. Asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, interacts with gastric oxidative metabolism and enhances stress-induced gastric lesions. *J. Physiol. Pharmacol.*, **63**(5):515-524.

Lafontan, M., Barbe, P., Galitzky, J., *et al*., 1997. Adrenergic regulation of adipocyte metabolism. *Hum. Reprod*., **12**(Suppl. 1):6-20. http://dx.doi.org/10.1093/humrep/12.suppl_1.6

Lin, H., Yuan, K.M., Zhou, H.Y., *et al*., 2014. Time-course changes of steroidogenic gene expression and steroidogenesis of rat Leydig cells after acute immobilization stress. *Int. J. Mol. Sci.*, **15**(11):21028-21044. http://dx.doi.org/10.3390/ijms151121028

Nathan, S.G., 1986. The epidemiology of the DSM-III psychosexual dysfunctions. *J. Sex Marital Ther*., **12**(4):267-281. http://dx.doi.org/10.1080/00926238608415413

Orr, T.E., Mann, D.R., 1990. Effects of restraint stress on plasma LH and testosterone concentrations, Leydig cell LH/HCG receptors, and in vitro testicular steroidogenesis in adult rats. *Horm. Behav*., **24**(3):324-341. http://dx.doi.org/10.1016/0018-506X(90)90013-N

Prabsattroo, T., Wattanathorn, J., Iamsaard, S., *et al*., 2015. *Moringa oleifera* extract enhances sexual performance in stressed rats. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **16**(3):179-190. http://dx.doi.org/10.1631/jzus.B1400197

Rai, J., Pandey, S.N., Srivastava, R.K., 2003. Effect of immobilization stress on spermatogenesis of albino rats. *J. Anat. Soc. India*, **52**(1):55-57.

Rai, J., Pandey S.N., Srivastava, R.K., 2004. Testosterone hormone level in albino rats following restraint stress of long duration. *J. Anat. Soc. India*, **53**(1):17-19.

Rao, M., Zhao, X.L., Yang, J., *et al*., 2015. Effect of transient scrotal hyperthermia on sperm parameters, seminal plasma biochemical markers, and oxidative stress in men. *Asian J. Androl.*, **17**(4):668-675. http://dx.doi.org/10.4103/1008-682X.146967

Retana-Márquez, S., Bonilla-Jaime, H., Vazquez-Palacios, G., *et al*., 2003. Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. *Horm. Behav.*, **44**(4):327-337. http://dx.doi.org/10.1016/j.yhbeh.2003.04.001

Sakr, S.A., Zowail, M.E., Marzouk, A.M., 2014. Effect of saffron (*Crocus sativus* L.) on sodium valporate induced cytogenetic and testicular alterations in albino rats. *Anat. Cell Biol*., **47**(3):171-179. http://dx.doi.org/10.5115/acb.2014.47.3.171

Scherer, I.J., Holmes, P.V., Harris, R.B., 2011. The importance of corticosterone in mediating restraint-induced weight loss in rats. *Physiol. Behav.*, **102**(2):225-233. http://dx.doi.org/10.1016/j.physbeh.2010.11.014

Ullrich, A., Schlessinger, J., 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**(2):203-212. http://dx.doi.org/10.1016/0092-8674(90)90801-K

Visconti, P.E., Kopf, G.S., 1998. Regulation of protein phosphorylation during sperm capacitation. *Biol. Reprod*., **59**(1):1-6. http://dx.doi.org/10.1095/biolreprod59.1.1

Warzecha, Z., Dembinski, A., Ceranowicz, P., *et al*., 2011. Role of sensory nerves in gastroprotective effect of anandamide in rats. *J. Physiol. Pharmacol.*, **62**(2):207-217.

Weissman, B.A., Sottas, C.M., Holmes, M., *et al*., 2009. Normal responses to restraint stress in mice lacking the gene for neuronal nitric oxide synthase. *J. Androl.*, **30**(5):614-620. http://dx.doi.org/10.2164/jandrol.108.007443

Wyrobek, A.J., Bruce, W.R., 1975. Chemical induction of sperm abnormalities in mice. *PNAS*, **72**(11):4425-4429.

http://dx.doi.org/10.1073/pnas.72.11.4425

Xu, M., Wei, Q., Zheng, K., *et al*., 2014. Protective effects of Big-leaf mulberry and physiological roles of nitric oxide synthases in the testis of mice following water immersion and restraint stress. *Acta Histochem.*, **116**(8):1323-1330. http://dx.doi.org/10.1016/j.acthis.2014.08.003

Yanagimachi, R., 1994. Mammalian fertilization. *In*: Knobil, E. (Ed.), The Physiology of Reproduction. Raven Press, New York, p.189-317.

Zardooz, H., Zahedi Asl, S., Gharib Naseri, M.K., *et al*., 2006. Effect of chronic restraint stress on carbohydrate metabolism in rat. *Physiol. Behav.*, **89**(3):373-378. http://dx.doi.org/10.1016/j.physbeh.2006.06.023

Zayachkivska, O.S., Gzhegotsky, M.R., Terletska, O.I., *et al*., 2006. Influence of *Viburnum opulus* proanthocyanidins on stress-induced gastrointestinal mucosal damage. *J. Physiol. Pharmacol*., **5**:155-167.

Zhang, M.H., Shi, Z.D., Yu, J.C., *et al*., 2015. Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-spicific proteinases 3 changes in fertile men. *J. Assist. Reprod. Genet*., **32**(5):747-755. http://dx.doi.org/10.1007/s10815-015-0451-0

中文概要

题　目：束缚应激对雄性大鼠睾丸磷酸化蛋白表达的影响

目　的：寻找雄性大鼠应激模型中潜在的睾丸蛋白标记物，探索评估男性生殖能力新的参数。

创新点：压力可以影响男性生育能力，但其具体机制尚未明了。睾丸组织中酪氨酸磷酸化蛋白在精子形成过程中扮演着重要的角色。本研究首次利用动物模型说明了压力对睾丸组织中酪氨酸磷酸化蛋白表达的影响，进而探索评估男性生殖能力新的参数。

方　法：雄性 SD 大鼠被随机分为对照组和束缚应激的实验组。实验组大鼠每天禁足 12 小时，持续 7 天。观察比较两组类固醇激素、精子密度、生殖系统形态学和组织学的差别，同时利用免疫印迹方法比较两组睾丸组织裂解产物中急性类固醇调节蛋白和酪氨酸的表达差别（早期在支持细胞中表达，后期在精子细胞中表达）。

结　果：实验组大鼠的睾酮水平和精子密度较对照组均显著降低，而精子畸形率和皮质酮显著升高。组织学比较发现实验组大鼠精子团数量减少并且出现输精管萎缩。两组大鼠睾丸组织中酪氨酸磷酸化蛋白的表达具有显著差异，实验组 95 kDa 的磷酸化蛋白表达量显著降低，但两组急性类固醇调节蛋白的表达没有显著差异。

结　论：本研究证明束缚应激可使大鼠睾丸组织磷酸化蛋白表达降低，并相应伴随着生殖能力参数的降低。这也许可以从一个方面解释压力对男性不育的影响。

关键词：束缚应激大鼠；类固醇激素合成急性调节蛋白；睾丸磷酸化蛋白

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