Histone deacetylase inhibitors suppress transdifferentiation of gonadotrophs to prolactin cells and proliferation of prolactin cells induced by diethylstilbestrol in male mouse pituitary

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Abstract

Diethylstilbestrol (DES), an estrogen agonist, increases prolactin (PRL) cells through transdifferentiation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) cells to PRL cells as well as proliferation of PRL cells in adult male mouse pituitary. Since hyperacetylation of histone H3 is implicated in the regulation of activation of various genes, we examined the effect of DES on the state of histone H3 acetylation. DES significantly reduced the immunohistochemical signal for acetylated histone H3 at lysine 9 (H3K9ac) in PRL, LH and FSH cells, but not for H3K18ac or H3K23ac. DES-treated mice were injected intraperitoneally with HDAC inhibitors (HDACi), sodium phenylbutyrate (NaPB) or valproic acid (VPA), to mimic the acetylation level of histone H3. As expected, HDACi treatment restored the level of H3K9ac expression in these cells, and also inhibited DES-induced increase in PRL cells. Furthermore, NaPB and VPA also abrogated the effects of DES on the population density of both LH and FSH cells. Similarly, the numbers of proliferating and apoptotic cells in the pituitary in NaPB- or VPA-treated mice were comparable to those of the control mice. Considered together, these results indicated that the acetylation level of histone H3 plays an important role in DES-induced transdifferentiation of LH to PRL cells as well as proliferation of PRL cells.

Key words: Diethylstibesterol; Prolactin; Gonadotrophs; Transdifferentiation; Histone H3 Lysine 9 acetylation; Histone deacetylase inhibitor.

Introduction

Diethylstilbestrol (DES), a synthetic nonsteroidal estrogen, induces reproductive tract anomalies, infertility and malignancy in human (McLachlan, Newbold and Bullock 1980; Alwis et al. 2011; Huo et al. 2017). In the pituitary gland of rodents, DES is known to increase the number of prolactin (PRL) cells and induce the development of prolactinoma (Cauwenberge et al. 2001; Matsubara, Harigaya and Nogami 2001; Ramadhani et al. 2015). We found previously that DES does not only induce proliferation of PRL cells, but also promotes transdifferentiation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) cells into PRL cells through the action of ERα (Shukuwa et al. 2006). However, the mechanism underlying DES effect on the population change of pituitary cells is largely unknown yet.

DES is known to act through binding to ERα and ERα is considered to modify chromatin structure by changing the acetylation state of lysine residues in histones (Fiskus et al. 2007; Azuma et al. 2009). In fact, ERα associates with acetyltransferases, including p300/CBP and p300CBP-associated factor (Stossi, Madak-Erdogan and Katzenellenbogen 2009). Moreover, ER coactivators; SRC1 and AIB1, possess histone acetyltransferase activity, while ERα mediates deacetylation by recruitment of histone deacetylases (Leader et al. 2006; Pradhan et al. 2012).

Histone acetylation is generally related to the activation of gene transcription, while the deacetylation induces gene silencing (Kuo and Allis 1998; Verdone, Caserta and Mauro 2005). Among the histones, histone H3 seems to be the most important in the regulation of genes that control cellular proliferation, differentiation and apoptosis (Strasak et al. 2009; Vempati et al. 2010; Hezroni et al. 2011; Qiao et al. 2015). For instance, acetylation of H3K9ac, H3K14ac and H3K27ac is involved in the transdifferentiation of bone marrow stem cells to hepatocytes (Liao et al. 2015). H3K9ac and H3K14ac are essential in transdifferentiation of B lymphocytes to macrophages (Barneda-Zahonero et al. 2012). Moreover, we have reported that histone H3 acetylation (H3K9ac, H3K18ac and H3K23ac) plays an important role in germ cell differentiation during mouse spermatogenesis (Dai et al. 2015; Song et al. 2011).

To mimic the level of histone acetylation, HDAC inhibitors (HDACi) are often used in a variety of cell differentiation experiments. Sodium phenylbutyrate (NaPB) is a salt of a short-chain fatty acid and is used for the treatment of urea cycle disorders (Iannitti and Palmieri 2011; AL-Keilani and Alsmadi 2018). Valproic acid (VPA) is also a short-chain fatty acid reported to be effective in the treatment of leukemias and other malignancies (Gottlicher et al. 2001; Lagace et al. 2004). NaPB and VPA are HDACi and reported to affect cell differentiation, growth arrest, and apoptosis of various cells including cancer cells through inhibition of histone

deacetylation (Karasawa and Okisaka 2004; Li et al. 2004; Liu et al. 2006; Merzvinskyte et al. 2006; Yoo et al. 2006; Gurvich et al. 2004; Duenas-Gonzalez et al. 2008; Dong et al. 2013; Mannaerts et al. 2010).

Based on the above findings, we postulated in the present study the involvement of changes in histone H3 acetylation in DES-induced transdifferentiation of LH and FSH cells to PRL cells. First, we examined the acetylation level of histone H3 in DES-treated mouse pituitary by immunohistochemistry and found that exposure to DES reduced the expression of H3K9ac in LH, FSH and PRL cells. Next, we administered either NaPB or VPA intraperitoneally in DES-treated mice and found that treatment with both HDACi neutralized the effects of DES on the expression of H3K9ac and the population of PRL, LH and FSH cells.

Materials and methods

Chemicals and biochemical

DES was purchased from ICN Biomedical (Aurora, OH). Sodium phenylbutyrate (NaPB), Tris [hydroxymethyl] aminomethane (Tris), bovine serum albumin (BSA; minimum 98%, γ-globulin free), dimethyl sulfoxide (DMSO), proteinase K, Brij 35 were purchased from Sigma-Aldrich (St Louis, MO). Paraformaldehyde (PFA) was from Merck (Darmstadt, Germany), 3, 3'- diaminobenzidine–4 HCl (DAB) was purchased from Dojin Chemicals (Kumamoto, Japan), 4-Cl-l-naphthol was from Tokyo Kasei Kogyo (Tokyo, Japan), biotin-16-dUTP and terminal deoxynucleotidyl transferase (TdT) were from Roche Diagnostics (Mannheim, Germany). Permount was obtained from Thermo Fisher Scientific (Hudson, NH). VPA and all other reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) and were of analytical grade.

Animals and treatment groups

Eight-week-old male ICR mice (Crj: CD-1) weighing 30-33 g were used. All experiments were conducted according to the principles and procedures outlined in the guidelines for animal experimentation of Nagasaki University with the approval of the institutional Animal Care and Use Committee (#1004010843). DES (20 mg/kg body weight) was dissolved in corn oil containing 5% ethanol. VPA at 300 mg/kg (Tremolizzo et al. 2002) and NaPB at 800 mg/kg (Dai et al. 2015) were dissolved in DMSO and phosphate-buffered saline (PBS; pH 7.4), respectively. To explore the involvement of histone H3 in DES induced cell population changes, the mice were injected subcutaneously with DES or corn oil at days 0, 5, 10, 15 and sacrificed at day 20 according to the protocol described in our previous study (Shukuwa et al. 2006). To examine the effects of NaPB and VPA, DES or corn oil was injected subcutaneously at days 0, 5 and 10. In the same group of mice, NaPB, VPA or PBS, DMSO as vehicle respectively was injected intraperitoneally every day from day 5 to day 14. All mice were sacrificed at day 10 or 15 and their pituitary glands were harvested (n=3 in each group). Mice treated with corn oil alone and injected with the vehicle (PBS) or NaPB were considered the control or NaPB group, respectively, while the DES-treated mice together with the vehicle or NaPB were considered the DES or DES+NaPB group, respectively. In the case of VPA, similar abbreviations were used, including the control, VPA, DES and DES+VPA groups.

Tissue preparation

The pituitary glands were fixed in 4% PFA in PBS at room temperature (RT) for about 17 hours and embedded in paraffin using a standard procedure.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (Song et al. 2011). Table 1 and 2 list the primary and secondary antibodies and their concentrations used in this study, respectively. Paraffin-embedded tissues were cut into 5-μm thick sections and placed onto silane-coated glass slides. For detection of the signals for H3K9ac, H3K18ac and H3K23ac, the sections were autoclaved at 120°C for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval after deparaffinization and rehydration. For detection of PRL, LH, FSH, the sections were processed in manner similar to that described above, except for the autoclave step. After inhibition of endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 15 min at RT, the sections were preincubated with 500 μg/ml normal goat IgG and 1% BSA in PBS for 1 h at RT to block nonspecific binding of antibodies. The sections were then reacted with the primary antibodies overnight at RT. After washing with 0.075% Brij 35 in PBS, they were reacted with HRP-goat anti-rabbit IgG for 1 h at RT. After washing in 0.075% Brij 35 in PBS, the sites of HRP were visualized with DAB, Ni, Co, and H_2O_2 for H3K9ac, H3K18ac and H3K23ac and with DAB and H_2O_2 counterstained with methyl green for PRL, LH and FSH. As a negative control, normal mouse or rabbit IgG was used at the same concentration instead of the primary antibodies in every experiment.

For simultaneous detection of PRL or LH and PCNA, we performed double staining, as described previously (An, Hishikawa and Koji 2005; Kawano et al. 2004). Briefly, the sections were stained with anti-PRL or anti-LH antibodies, and HRP sites were visualized with DAB and H_2O_2. Then, the sections were reacted with anti-PCNA antibody and HRP sites were visualized with 4-Cl-1-naphthol and H_2O_2 solution. For simultaneous detection of PRL and LH, the PRL signal was first visualized with DAB and H_2O_2 and LH signal was visualized with 4-Cl-l-naphthol and H_2O_2 solution. The negative control for double immunohistochemistry, normal mouse or rabbit IgG was used at the same concentration for each primary antibody instead of the primary antibodies in every experiment.

[Please insert Tables 1 and 2 here]

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL was performed using the method described previously (Dai et al. 2016). Paraffin sections were dewaxed and digested with 10 μg/ml of proteinase K in PBS at 37 °C for 15 min. Then, the sections were reacted with 1× TdT buffer (825 mM Tris/HCl buffer, pH 6.6, containing 0.2 M potassium cacodylate and 0.25 mg/ml BSA) alone at RT for 30 min. After the incubation, the slides were reacted with 800 U/ml TdT dissolved in TdT buffer supplemented with 0.5 μM biotin-16-dUTP, 20 μM dATP, 1.5 mM $CoCl_2$, and 0.1 mM dithiothreitol at 37 °C for 90 min. As a negative control, TdT reaction was conducted without TdT. After washing with Milli-Q water, HRP-goat anti-biotin antibody was applied on the sections and then washed. The sites of HRP were visualized by a mixture of DAB, Ni, Co, and H_2O_2, as described above.

Quantitative analysis

The results of immunohistochemistry were graded as positive or negative, compared to that of the negative control. For counting the number of PRL, LH and FSH cells, all microscopic fields of each specimen were photographed (x20 magnification), printed out, and shuffled to avoid bias. The number of positive cells was counted by two investigators. On average, the total number of cells counted in each specimen was more than 40,000 cells. The number of positive cells was expressed as the percentage of positive cells per total number of counted cells.

The signal intensity of H3K9ac (200 nuclei) was measured in randomly selected fields at 400× magnification (5 fields) using an image analyzer (DAB analysis system, Germany). The positive nuclei were evaluated based on the staining density relative to the level of staining using the same concentration of each IgG. The sum of the pixel values in each nucleus was measured and the average pixels in a nucleus was calculated as signal intensity. The results were expressed as a ratio of signal intensity in the test group to that of the control group, which were administrated corn oil and vehicle of each HDACi. The double-positive cells of PCNA and PRL cells were expressed as the percentage of PCNA-positive cells in PRL cells.

Statistical analysis

All data were expressed as mean±SD. Differences between groups were examined for statistical significance using the Student's t-test and ANOVA following multiple comparisons. A *P* value less than 0.05 denoted the presence of a statistically significant difference. All analyses were performed with a statistical software package Statcel 4 (OMS, Saitama, Japan).

Results

Effects of DES on acetylation levels of histone H3 in mouse pituitary

To examine the effects of DES on acetylation of histone H3, we immunohistochemically compared the expression levels of histone H3K9ac, H3K18ac and H3K23ac in the pituitary of mice treated with corn oil or DES (Fig. 1). Strong staining for H3K9ac, H3K18ac and H3K23ac was detected in the nuclei of the anterior pituitary cells in the control, while application of the same concentration of normal rabbit IgG as each antibody presented no signal (Fig. 1a and 1b). DES-treated mice showed markedly weak staining for H3K9ac in the pituitary, but comparable staining for H3K18ac and H3K23ac (Fig. 1c).

[Please insert Fig. 1 here]

Effects of DES and DES plus NaPB on acetylation levels of H3K9 in PRL, LH and FSH cells

To clarify the effects of DES on the acetylation of H3K9 in individual cells producing different hormones, we conducted immunohistochemistry for PRL, LH and FSH in the exactly adjacent sections to that used for H3K9ac. Moreover, we examined whether NaPB can mimic the level of acetylation as HDACi or not. As shown in Fig. 2a, the signal for H3K9ac in PRL, LH and FSH cells in DES treated mice were similarly reduced compared to those of the control and NaPB mice, whereas that of DES+NaPB mice was similar to the control level. No staining was found in sections reacted with normal rabbit IgG as a negative control for PRL, LH and FSH (Fig. 2b). Quantitative analysis of the signal density of H3K9ac per cell showed that DES significantly reduced the signal of H3K9ac in PRL, LH and FSH cells, but NaPB treatment neutralized the effect of DES (Fig. 2c).

[Please insert Fig. 2 here]

Effects of NaPB on PRL, LH and FSH cell population in DES-treated mouse pituitary

In parallel with the examination for the expression of acetylated histone H3, we also analyzed the effects of NaPB on PRL, LH and FSH cell populations in DES-treated mice (Fig. 3). In agreement with the results of our previous study (Shukuwa et al., 2006), treatment with DES significantly increased the number of PRL cells, but decreased those of LH and FSH cells, compared to NaPB group. There was no significant different between control and NaPB group. Interestingly, however, NaPB treatment abrogated the effects of DES and the population of these PRL, LH and FSH cells was almost similar to that of the NaPB group.

[Please insert Fig. 3 here]

Effects of VPA on acetylation levels of H3K9 in PRL, LH and FSH cells

Since each HDACi has various unique side effects, we performed similar experiments using VPA, another HDACi. As shown in Fig. 4, immunohistochemistry and the following quantitative analysis revealed that VPA also abrogated the effects of DES on the expression of H3K9ac, similar to that of NaPB. Unexpectedly, however, VPA alone significantly decreased the expression of H3K9ac in all PRL, LH and FSH cells. Pituitary sections reacted with normal rabbit IgG as a negative control for H3K9ac, PRL, LH and FSH were blank as shown in Fig. 1b and 2b.

[Please insert Fig. 4 here]

Effects of VPA on PRL, LH and FSH cell population in DES-treated mouse pituitary

To determine whether VPA has similar effect to NaPB on PRL, LH and FSH cell populations, we performed immunohistochemistry of PRL, LH and FSH cells and the number of each cells was counted. The numbers of PRL, LH and FSH cells in the DES+VPA mice were similar to those of the VPA group (Fig. 5).

[Please insert Fig. 5 here]

Effects of VPA on ROS production in pituitary cells

VPA reduced the acetylation level of H3K9 compared to control (Fig.4). To gain some additional insights into the unexpected effect, we conducted immunohistochemistry for ROS marker, 4-hydroxynonenal (4-HNE), As shown in Fig. 6a, immunostaining for 4-HNE was detected in the cytoplasm of pituitary cells only in the VPA mice, although the signal was not detected in the control and NaPB-treated mice. The sections reacted with normal rabbit IgG as a negative control for 4-HNE showed no staining (Fig. 6b).

[Please insert Fig. 6 here]

Effects of NaPB and VPA on proliferation and apoptosis of PRL and LH cells in DES-treated mice

The above results indicated that HDACi restored the number of PRL and LH cells to the control level, and could have neutralized the effects of DES. To examine the role of cell proliferation and apoptosis on the results, we conducted immunohistochemistry for PCNA and TUNEL staining respectively (Fig. 7). PCNA-positive PRL cells were significantly increased in DES-treated mice compared to the control, DES+HDACi and HDACi mice

(Fig. 7a-d). In the case of LH cells, PCNA-positive nuclei were rarely detected (Fig. 7e and f). Apoptotic cells were also hardly detected in all groups (Fig. 7g and h).

[Please insert Fig. 7 here]

Effects of NaPB on transdifferentiation of LH cells to PRL cells in DES-treated mice

We reported previously the presence of PRL and LH double-positive cells at days 5-10 after DES treatment (Shukuwa et al. 2006). To examine whether NaPB affects the transdifferentiation of LH cells to PRL cells or not, we performed double immunohistochemistry using mouse pituitary sections obtained at 10 days after DES treatment by using antibodies for PRL and LH. Double-positive cells for PRL and LH were seen in the DES mice, whereas no double-positive cells were detected in DES+NaPB mice (Fig. 8a). As a negative control, the same concentration of normal rabbit IgG was applied instead of each primary antibody on pituitary of DES+NaPB mice which showed no staining (Fig. 8b).

[Please insert Fig. 8 here]

Discussion

In the present study, we confirmed the transdifferentiation of LH and FSH cells into PRL cells and stimulation of PRL cell proliferation by DES in male mouse pituitary and found a significant decrease in the level of histone H3K9 acetylation in these gonadotrophs as well as PRL cells. To clarify the role of histone H3K9 acetylation in the observed action of DES, we investigated the effects of HDACi on the expression of H3K9ac and the kinetics of PRL cells *in vivo*. The results showed that the effect of DES on PRL cell population was almost completely abrogated by HDACi, strongly indicating the essential roles of epigenetic factors in the maintenance of differentiation states of various hormonal cells in mammalian anterior pituitary.

In fact, the relationship between estrogen and acetylation of histones has been demonstrated in various experimental systems, though it seems to be highly controversial. Nye et al reported that estradiol promoted acetylation of histone H3 and H4 in A03_1 CHO DG44 cells (Nye et al. 2002), whereas in rat pituitary cell line (GH4 cells), estrogen increased histone H4 acetylation, but not that of histone H3 in the PRL promoter region (Liu et al. 2005). Also, it has been reported that estradiol increased acetylation level of histone H3 in memory-related hippocampus of female mouse (Fortress et al. 2014). In the MCF-7 human mammary cancer cell line, however, estradiol treatment decreased acetylation level of histone H3. Therefore, estrogen action on histone acetylation in cells and tissues seems to depend on different usages of histone acetyltransferases and HDACs (Roth, Denu and Allis 2001; Bolden, Peart and Johnstone 2006; Xu, Parmigiani and Marks 2007; Lane and Chabner 2009; Mottamal et al. 2015).

In the present study, we encountered differences in the actions of NaPB and VPA. Unlike NaPB, VPA alone significantly decreased the level of H3K9ac in PRL, LH and FSH cells. Various HDACi (Dai et al. 2015) including VPA (Kawai and Arinze 2006; Tung and Winn 2011) have already been shown to induce ROS. Since it was reported that ROS caused hypoacetylation in human hepatoma cell line (Hep3B) (Kang et al. 2003), probably through the activation of HDAC as was shown in rat renal cells (Noh et al. 2009), a similar phenomenon could occur in VPA treatment in the pituitary, involving VPA-insensitive HDAC. In fact, as shown in Fig. 6, 4-HNE, a marker for ROS action, was detected in the VPA alone group, but not NaPB one, perhaps providing an explanation for the decrease in histone H3K9ac. In the DES+VPA group, the effect of VPA alone could be overwhelmed by inhibition of DES.

In our previous work (Sakamoto et al. 2013), we also reported the lethal effects of the same HDACi on various transplanted tumors, which can be attributed to their effect on ROS production. Therefore, it is possible

that HDACi also have cytotoxic effects on pituitary cells. Nevertheless, the population of PRL, LH and FSH cells was not affected. The cytotoxic effects of these HDACi on these cells was minimal.

With regard to the effects of HDACi on PRL cell proliferation, both NaPB and VPA completely suppressed the increase in the number of PCNA-positive PRL cells induced by DES. HDACi have been reported to inhibit cell proliferation in cancer cells (Sakajiri et al. 2005; Damaskos et al. 2015; Hrgovic et al. 2016). Considering that administration of DES or estrogen was associated with the development of prolactinoma in rat and human pituitary glands (Mukdsi et al. 2004; Mucha et al. 2007; Chen et al. 2016; Fujiwara et al. 2017; Horiguchi et al. 2018), drugs related to the regulation of epigenomes, including HDACi, could potentially provide a new therapeutic approach.

In conclusion, the results of the present study indicated that DES seems to induce transdifferentiation of LH and FSH cells to PRL cells as well as proliferation of PRL cells through hypoacetylation of histone H3K9. In fact, HDACi effectively canceled the effects of DES. For a better understanding of the regulation of differentiation states of pituitary cells by acetylation of histones, approaches to correlate any change in histone modification with individual gene activity would be indispensable in future studies.

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Figure legends

Figure 1. Effects of DES on the expression of histone H3K9ac, H3K18ac and H3K23ac in mouse pituitary

(a) Immunohistochemistry for H3K9ac, H3K18ac or H3K23ac in pituitary of control and DES-treated mice. (b) As a negative control, pituitary sections of DES-treated mice were reacted with the same concentration of normal rabbit IgG as each antibody. Magnification, ×400. Scale bars, 50 μm. (c) Quantitative analysis of the signal intensity of H3K9ac, H3K18ac and H3K23ac in pituitary cells. Data are mean±SD. **P<0.01.

Figure 2. Effects of NaPB on the expression of histone H3K9ac in PRL, LH and FSH cells in pituitaries of control- and DES-treated mice.

(a) Immunohistochemistry for H3K9ac in PRL, LH and FSH using serial pituitary sections. Red arrowheads: signals of H3K9ac, black arrowheads: signals of PRL, LH or FSH. (b) As a negative control, sections of pituitaries in DES+NaPB mice were reacted with the same concentration of normal rabbit IgG instead of specific antibodies. Magnification, ×400. Scale bars, 50 μm. (c) Quantitative analysis of the signal intensity of H3K9ac in PRL, LH and FSH cells in pituitary of each group. Data are mean±SD. **P<0.01.

Figure 3. Effects of NaPB on PRL, LH and FSH cell population in pituitaries of control- and DES-treated mice.

PRL, LH and FSH cell counts based on the results shown in Fig. 2(a). Data represent mean±SD. *P<0.05, **P<0.01.

Figure 4. Effects of VPA on expression of histone H3K9ac in PRL, LH and FSH cells in pituitaries of control- and DES-treated mice.

(a) Immunohistochemistry for H3K9ac in PRL, LH and FSH using serial pituitary sections. Red arrowheads: signals of H3K9ac, black arrowheads: signals of PRL, LH or FSH. Magnification, ×400. Scale bars, 50 μm. (b) Quantitative analysis of the signal intensity of H3K9ac in PRL, LH and FSH cells. Data are mean±SD. *P<0.05, **P<0.01.

Figure 5. Effects of VPA on PRL, LH and FSH cell population in pituitaries of control- and DES-treated mice.

PRL, LH and FSH cell counts based on the results shown in Fig. 4(a). Data are mean±SD. *P<0.05, **P<0.01.

Figure 6. Expression of 4-HNE in pituitaries of control-, NaPB- and VPA-treated mice.

(a) Immunohistochemistry of 4-HNE positive cells in pituitaries of the vehicle-, NaPB- and VPA-treated mice. (b) As a negative control, the sections of NaPB-treated mice were reacted with the same concentration of normal rabbit IgG. Black arrowheads: signals of 4-HNE positive cells. Magnification, ×400. Scale bars, 50 μm

Figure 7. Effects of VPA on cell proliferation and apoptosis in pituitaries of control- and DES-treated mice.

(a) Double immunohistochemistry of PCNA (blue) and PRL (brown) in the pituitaries of control, NaPB, DES and DES+NaPB groups. Black arrowheads: double-positive cells. Magnification, ×400. Scale bars, 50 μm. (b) Percentage of PCNA-positive cells among PRL cells. Data are mean±SD. **P<0.01. (c) Double immunohistochemistry of PCNA (blue) and PRL (brown) in the pituitaries of control, VPA, DES and DES+VPA groups. Black arrowheads: double-positive cells. Magnification, ×400. Scale bars, 50 μm. (d) Percentage of PCNA-positive cells among PRL cells. Data are mean±SD. **P<0.01. (e) Double immunohistochemistry of PCNA (blue) and LH (brown) in pituitaries of DES and DES+NaPB groups. Red arrowheads: PCNA-positive cells, black arrowheads: LH-positive cells. Magnification, ×400. Scale bars, 50 μm. (f) Double immunohistochemistry of PCNA (blue) and LH (brown) in pituitaries of DES and DES+VPA mice. Red and black arrowheads highlight PCNA-positive cells and LH-positive cells, respectively. Magnification, ×400. Scale bars, 50 μm. (g) TUNEL staining in control, NaPB, DES and DES+NaPB groups. Black arrowheads: TUNEL-positive cells. Magnification, ×400. Scale bars, 50 μm. (h) TUNEL staining in control, VPA, DES and DES+VPA groups. Black arrowheads: TUNEL-positive cells. Magnification, ×400. Scale bars, 50 μm.

Figure 8. Effects of 10-day treatment with DES and DES+NaPB on expression of PRL and LH in pituitary cells.

(a) Double immunohistochemistry for PRL and LH in pituitaries of mice treated for 10 days with DES and DES+NaPB. Brown: PRL-positive cells, blue: LH-positive cells, black arrowheads: PRL-LH-double-positive cells. (b) As a negative control, the same concentration of normal rabbit IgG was applied instead of each primary antibody on pituitary of DES+NaPB mice. Magnification, ×400. Scale bars, 50 μm.

Table 1 Antibodies used in this experiment

Primary antibodies

Antibody	Host	Monoclonal/ Polyclonal	Supplier	Code	Dilution
PRL	Rabbit	Polyclonal	Biogenesis (Bournemouth, UK)	7770-0104	1:800
LH	Rabbit	Polyclonal	Biogenesis (Bournemouth, UK)	5720-8989	[a]1:1000
FSH	Rabbit	Polyclonal	Biogenesis (Bournemouth, UK)	4561-6959	[b]1:400
H3K9ac	Rabbit	Monoclonal	Cell Signaling Technology (Beverly, MA, USA)	9649	[c]1:1600
H3K18ac	Rabbit	Polyclonal	Cell Signaling Technology (Beverly, MA, USA)	9675	[c]1:100
H3K23ac	Rabbit	Polyclonal	Cell Signaling Technology (Beverly, MA, USA)	8848	[c]1:25
PCNA	Mouse	Monoclonal	DAKO (PC10, Glostrup, Denmark)	M0879	1:200
4-HNE	Mouse	Monoclonal	NOF Co. (Tokyo, Japan)	N213220	1:200

[a]Negligible cross-reactivity to FSH was described in the company instruction.

[b]No significant cross reaction to LH was reported (Katayama et al. 2000).

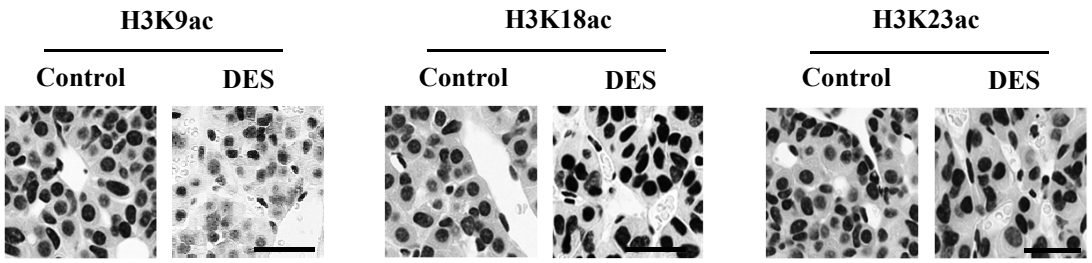
[c]The specificity of these antibodies was confirmed by the company with western blot and immunohistochemistry in each instruction manual.

Secondary antibodies

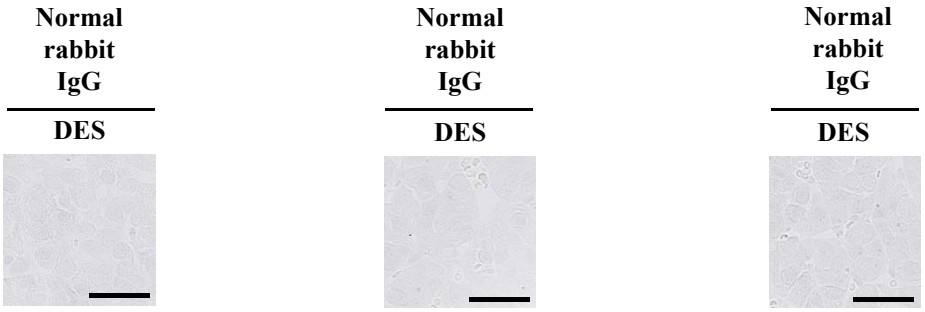
Antibody	Supplier	Dilution
HRP-goat anti-mouse IgG	Millipore (Temecula, CA, USA)	1:100
HRP-goat anti-rabbit IgG	Millipore (Temecula, CA, USA)	1:100
HRP-goat anti-biotin IgG	Vector Laboratories (Burlingame, CA, USA)	1:100

Figure 1 **Nandar Tun et al.**

a



b



c

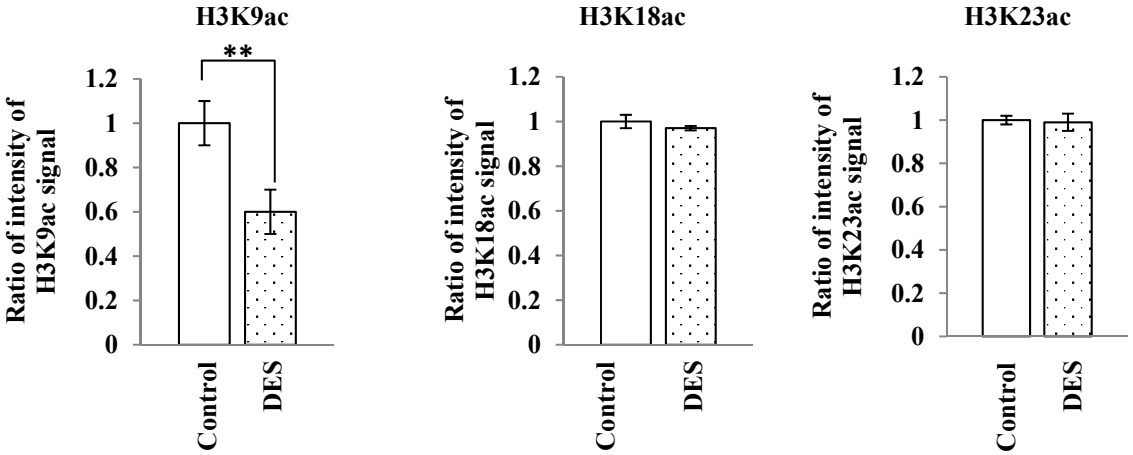


Figure 2

Nandar Tun et al.

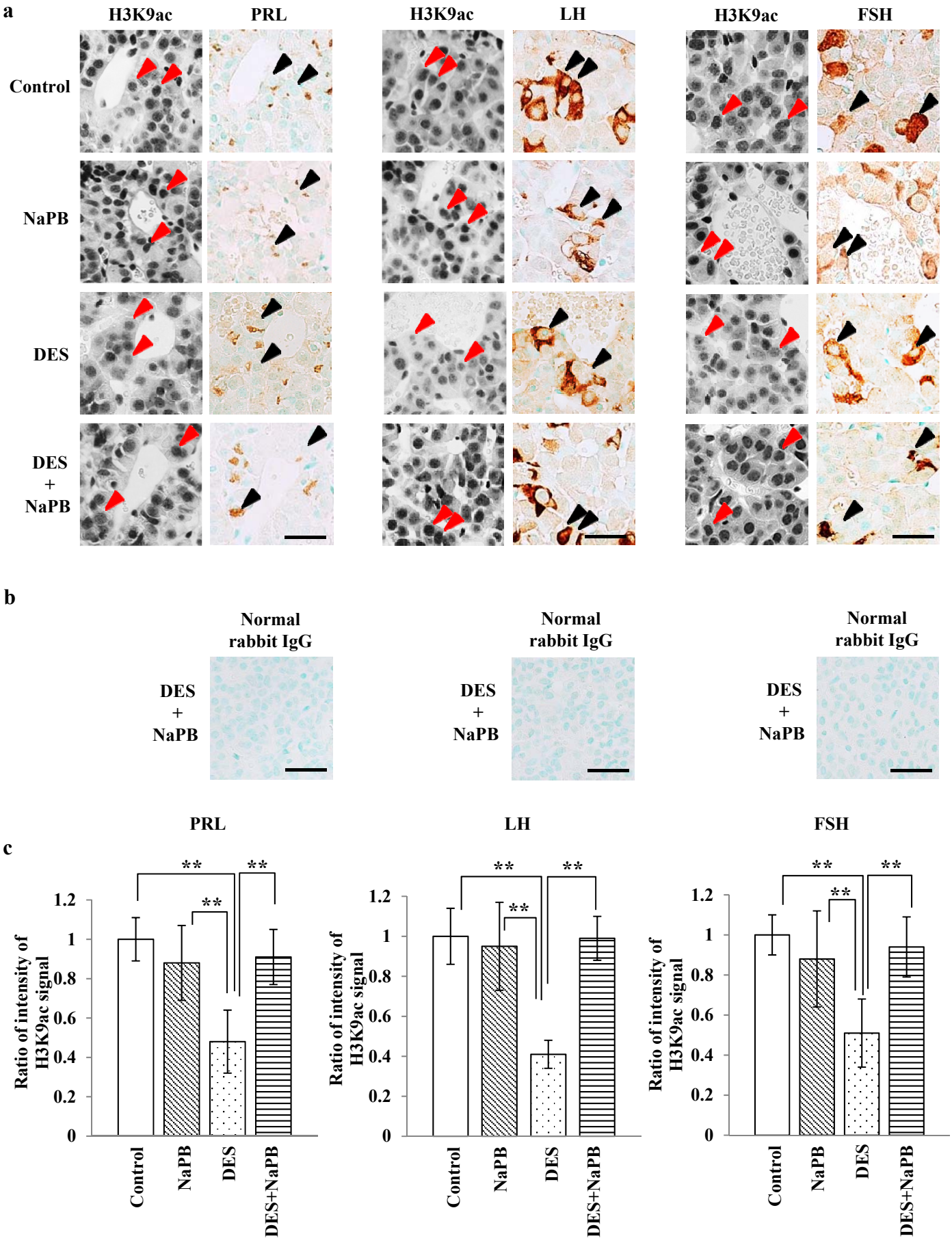
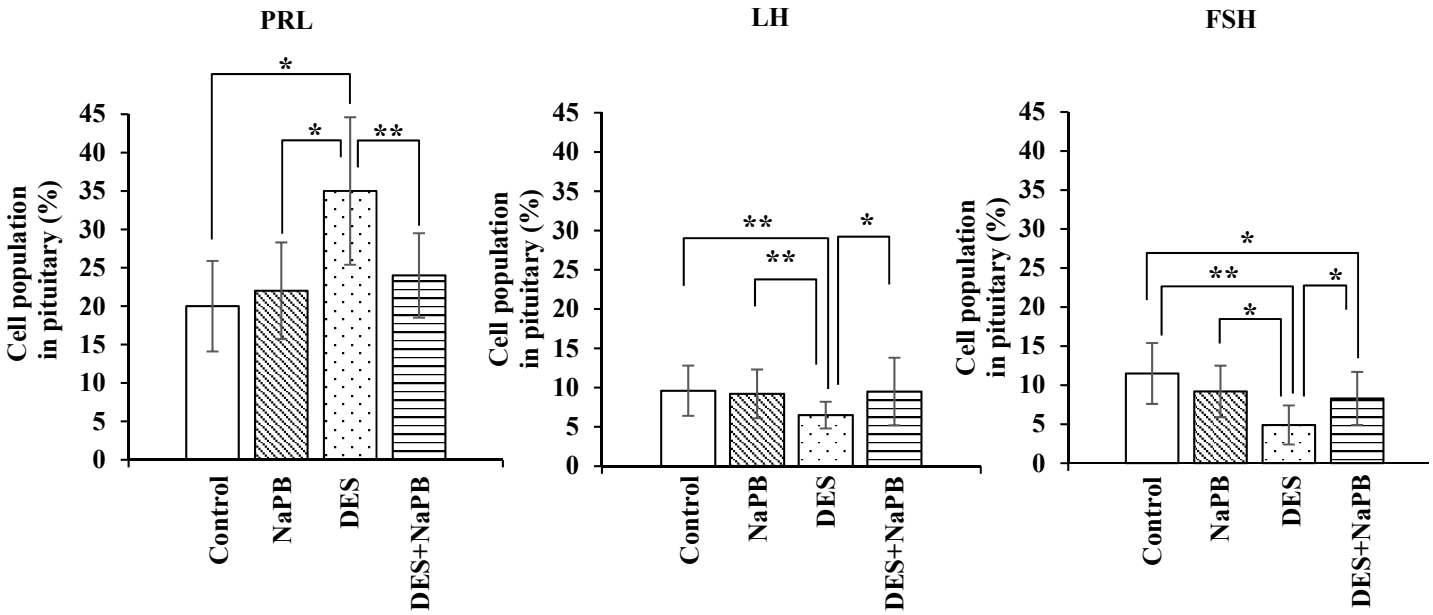
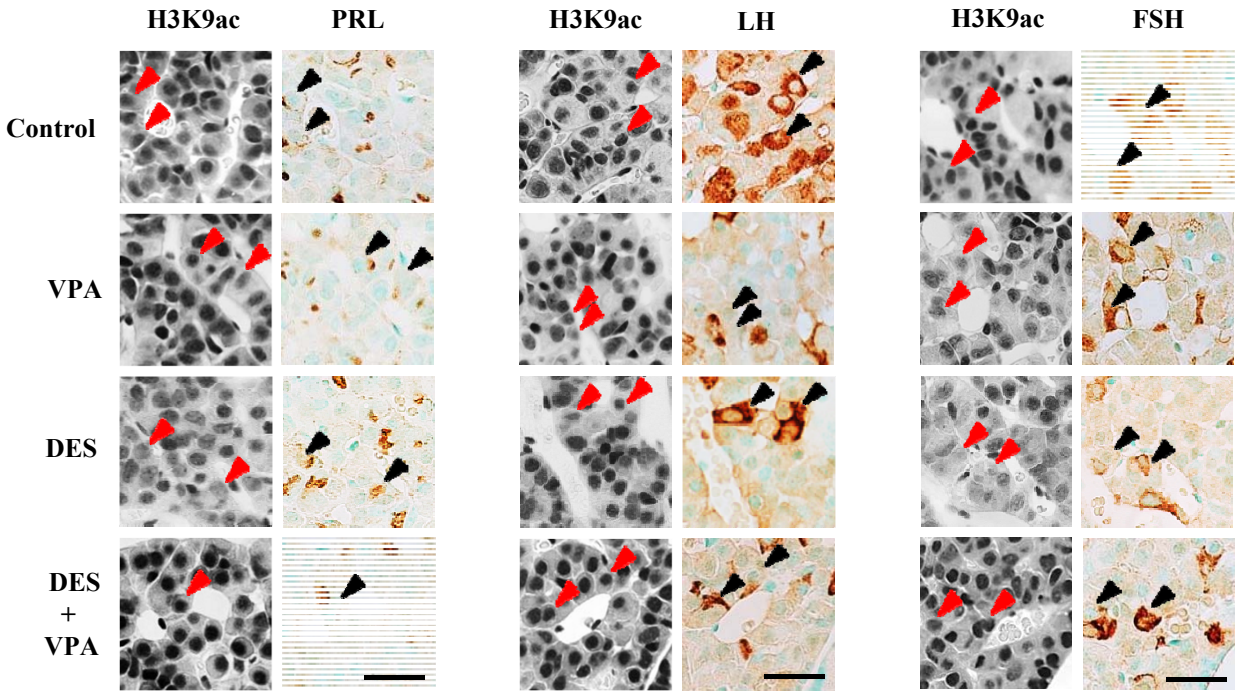


Figure 3 **Nandar Tun et al.**



a


H3K9ac
PRL
H3K9ac
LH
H3K9ac
FSH
Control
VPA
DES
DES + VPA

b

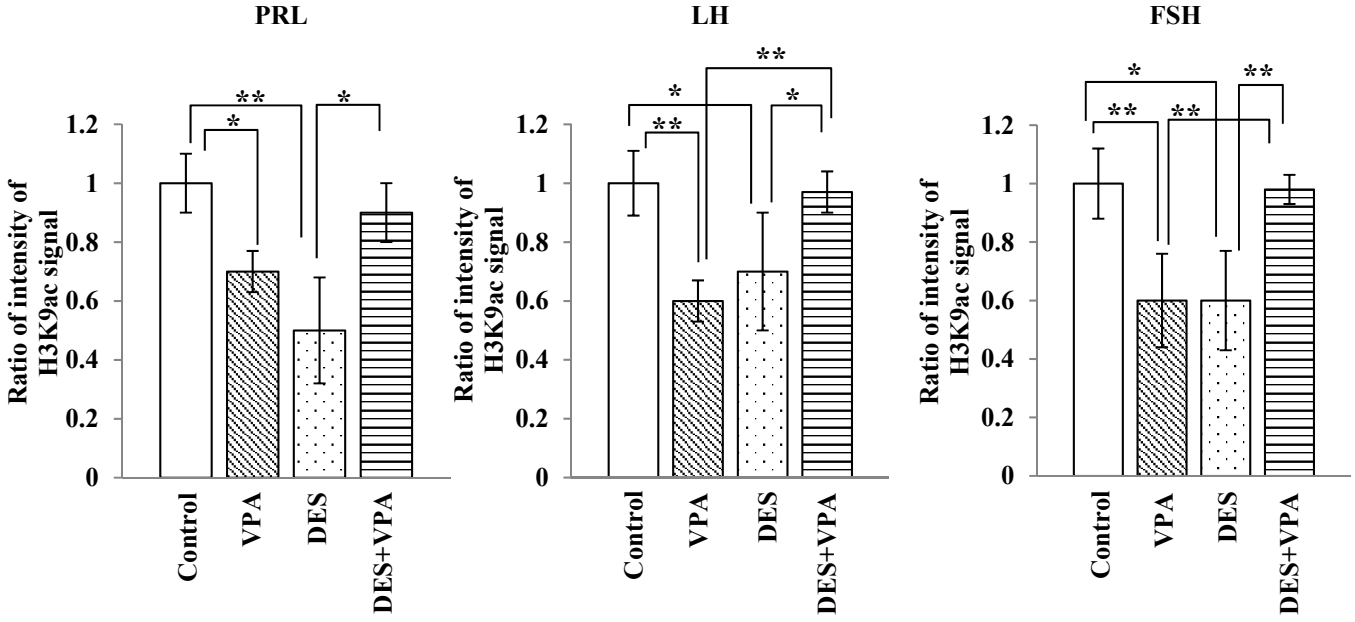

PRL
LH
FSH
Ratio of intensity of H3K9ac signal
0
0.2
0.4
0.6
0.8
1
1.2
Control
VPA
DES
DES+VPA
*
**

Figure 5 **Nandar Tun et al.**

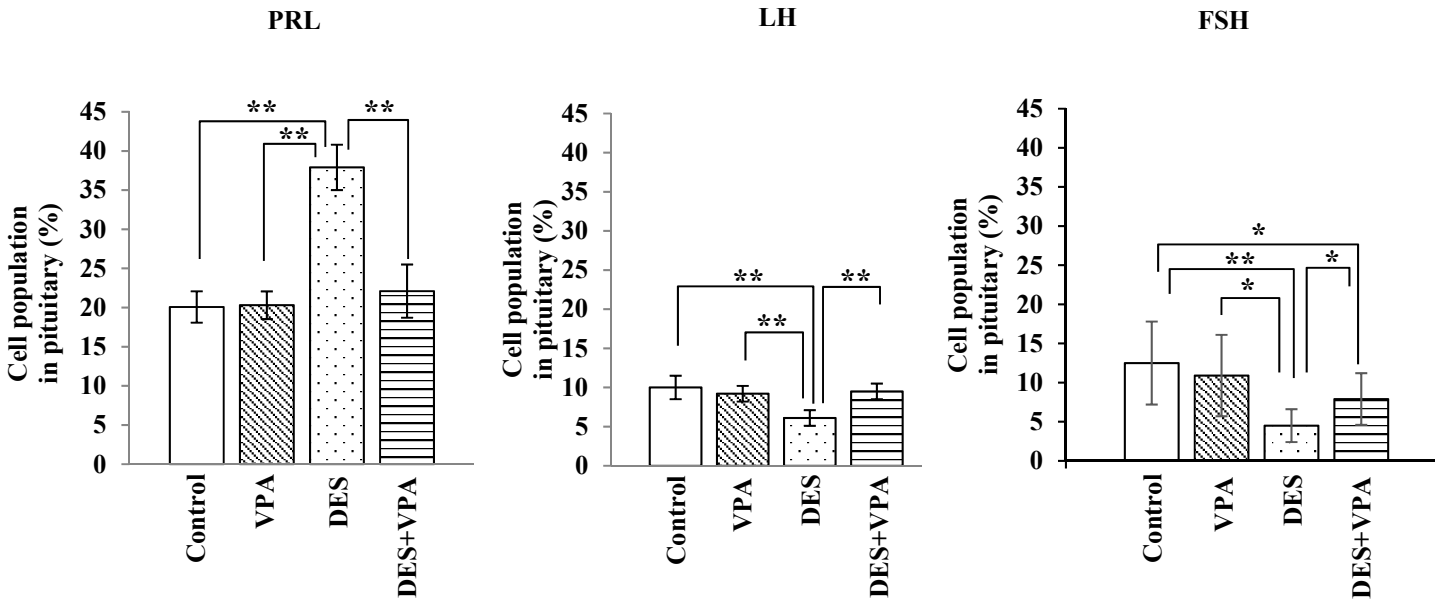
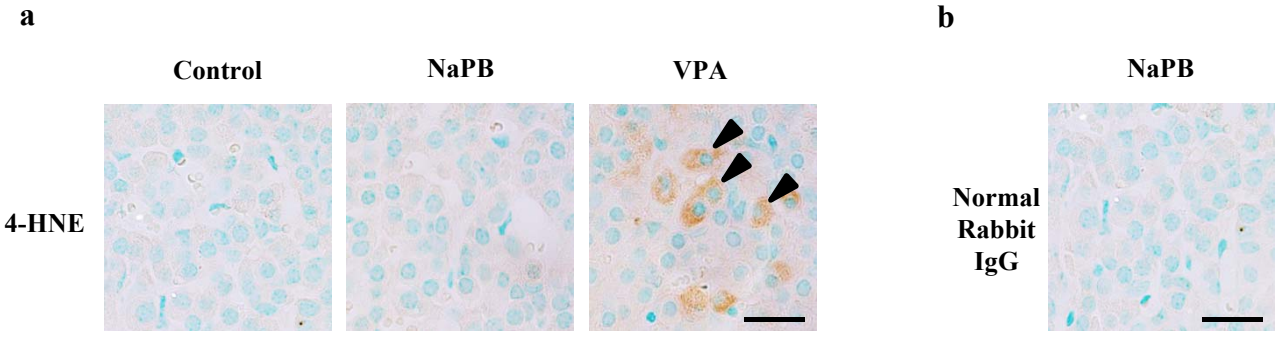


Figure 6 **Nandar Tun et al.**



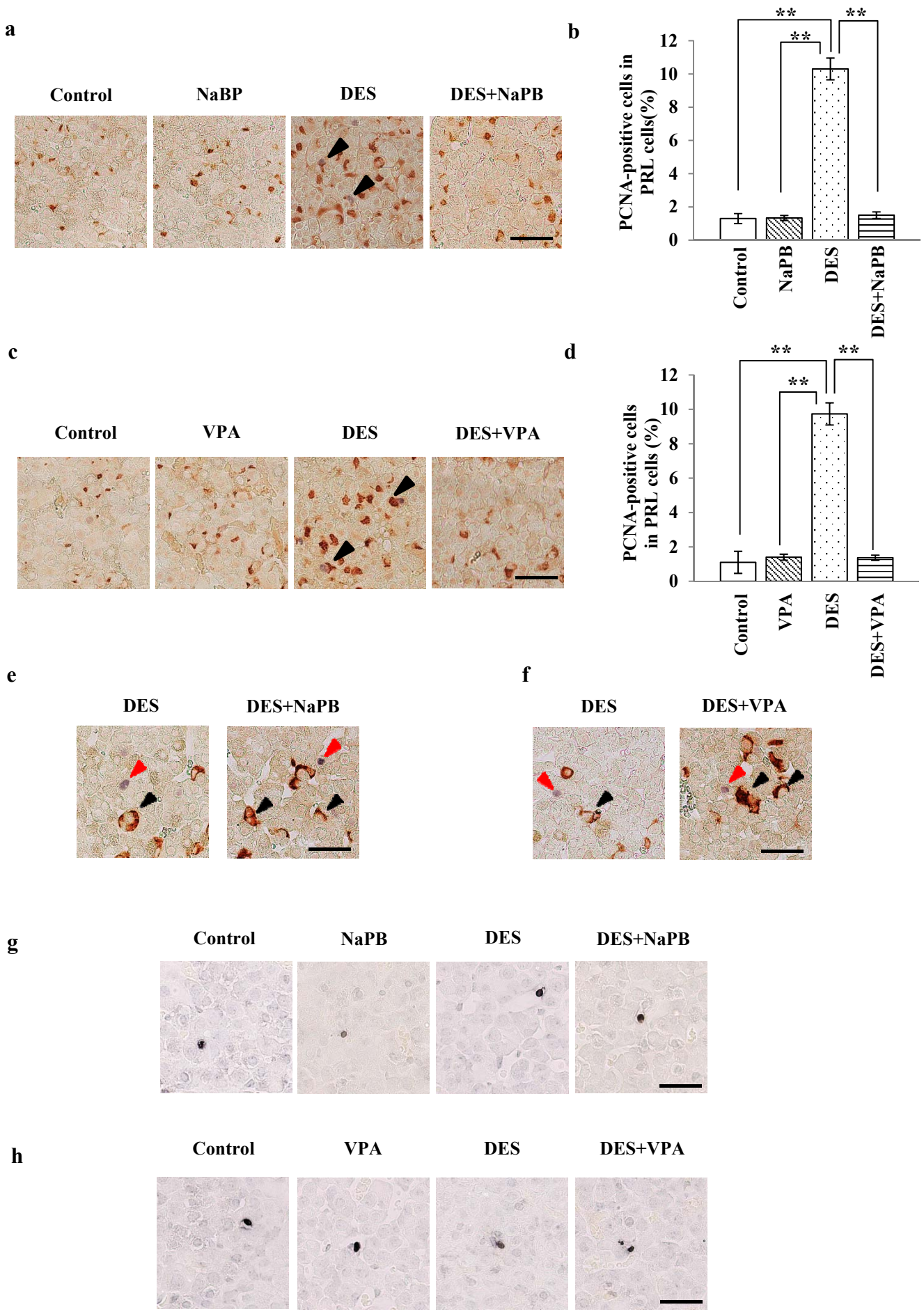

a
Control
NaBP
DES
DES+NaPB
b
PCNA-positive cells in PRL cells(%)
Control
NaPB
DES
DES+NaPB
c
Control
VPA
DES
DES+VPA
d
PCNA-positive cells in PRL cells (%)
Control
VPA
DES
DES+VPA
e
DES
DES+NaPB
f
DES
DES+VPA
g
Control
NaPB
DES
DES+NaPB
h
Control
VPA
DES
DES+VPA

Figure 8 **Nandar Tun et al.**

a **b**