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Histopathology and Changes of Pro-Caspase 3 and Androgen Receptor Expressions with Increased MDA in Epididymis of a Depressive-Like Behavior Rat Model

Histopatología y Cambios en la Expresión de la Procaspasa 3 y del Receptor de Andrógenos con el Aumento de MDA en el Epidídimo de un Modelo de Rata con Comportamiento Similar a la Depresión

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SUMMARY: Chronic stress (CS) with increased cortisol level is known as a risk factor to promote depressive-like behaviors and can cause many disorders including male infertility. Dexamethasone (DEXA), a synthetic corticosteroid medicine, is not only used to treat inflammation but also used to induce CS in animal models. Although DEXA has been shown to damage male reproductive parameters, its adverse effects on epididymis important for sperm maturation has never been reported. This study aimed to investigate the alterations of histology and protein expressions in epididymis induced with DEXA. Twenty adult male rats were divided into control and DEXA (1.5 mg/KgBW) groups by treating for 21 consecutive days (10 rats/ group). Chronic stress with depressive-like behaviors was determined with forced swimming and tail suspension tests. Histopathology, malondialdehyde (MDA) level, and expressions of pro-caspases (3 and 9), androgen receptor (AR), and heat shock protein-70 (Hsp-70) in epididymis were examined. Results showed that weigh and sperm mass of caudal epididymis were reduced in DEXA rats with some cribriform change and hyperplasia of clear cells. DEXA significantly increased the epididymal epithelial cell height, muscular wall thickness, and MDA levels but no difference in collagen fiber area compared to control. Significantly, the procaspase 3 expression was decreased and AR tended to be low expressed in DEXA epididymis tissue. No different expressions of pro-caspase 9 and Hsp-70 were observed between groups. In conclusion, DEXA induced histopathology of cauda epididymis via lipid oxidation and caspase 3 activation. It could be used to explain the functional immaturity of epididymal sperm in CS male with depressive-like behavior.

KEY WORDS: Chronic stress; Dexamethasone; Cauda epididymis; Malondialdehyde; Caspase 3.

INTRODUCTION

Depressive-like behavior caused from chronic stress (CS) is associated with increased glucocorticoid or cortisol levels via the hypothalamic-pituitary-adrenal axis (Nicolaides *et al*., 2015; Hong *et al*., 2021). The elevated glucocorticoids can suppress the immune system and inhibit the inflammation (Rhen & Cidlowski, 2005). Recently, the synthetic drugs in a group of glucocorticoids including dexamethasone (DEXA) have been used to treat the rheumatoid and osteoarthritis pains (Black & Grodzinsky, 2019). However, DEXA has been reported to affect the urinary, respiratory, muscular, skeletal, nervous, and reproductive systems (Oelzner *et al*., 2010; Hoes *et al*., 2015; Zhang *et al*., 2021; Alev *et al*., 2022). Moreover, the long-term use of DEXA for inflammatory treatments or its use for CS induction in experimental animal models has many side effects on male reproduction. Several studies have

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reported that CS induced by DEXA showed adverse effects on reproductive organs including the decrease of sperm qualities, concentration, semen volume, and protein changes via apoptotic pathway in humans and animals (Gür *et al*., 2005; Ozer Kaya *et al*., 2020). In the testis, DEXA was demonstrated to decrease the antioxidant enzyme activities and to increase the lipid peroxidation (Hasona, 2018; Jeje *et al*., 2020; Nassan *et al*., 2021; Saleh *et al*., 2022). It also could increase testicular apoptotic protein markers including Bax, caspase 3, p53, and BCL-2 levels (Mahmoud *et al*., 2009; Jeje *et al*., 2020; Nassan *et al*., 2021; Saleh *et al*., 2022). Furthermore, it has been shown that the spermatogenesis index range and testicular fructose levels of DEXA treated animals were significantly reduced (Aykan *et al*., 2020; Saleh *et al*., 2022).

Moreover, DEXA caused testicular histopathology such as vacuolation, degeneration, and nuclear hyperchromasia in rat spermatogonia (Nassan *et al*., 2021; Saleh *et al*., 2022). Sadeghzadeh *et al*. (2019) also reported the increased apoptosis in mouse seminiferous tubule induced with DEXA which was corroborated to the decrease of testis Johnsen scores. In hamsters, DEXA decreased sex hormone levels, testicular weight and Johnsen scores, antioxidant enzyme activities and the expressions of apoptotic proteins were increased in testicular lysate (Mukherjee *et al*., 2015). Interestingly, the weight of rat epididymis treated with DEXA was reported to be significantly decreased with low sperm quality (Silva *et al*., 2014; Jeje *et al*., 2017; Kempinas *et al*., 2019a; Jeje *et al*., 2020; Nassan *et al*., 2021; Azimi Zangabad *et al*., 2023). Since the epididymis is essential for functional sperm maturation, its effects of CS induced by DEXA on epididymis tissue have never been documented. Therefore, the aim of this study was to observe histopathology, apoptotic protein markers, and malondialdehyde (MDA) level in cauda epididymis of rats treated with DEXA to induce CS associated with depressive-like behaviors.

MATERIAL AND METHOD

Animals and experimental design. The 20-adult male Wistar rats were purchased from the Northeast Laboratory Animal Center, Khon Kean University. Rats were divided into two groups (n, 10/group): control and the dexamethasone (DEXA)-treated depression (chronic stress) like behavior groups. Control rats were injected with Na_3PO_4 and DEXA animals were injected intraperitoneally with a dose of 1.5 mg/kgBW for 21 consecutive days (Wu *et al*., 2021; Ueno *et al*., 2022). Before experiment, the tail suspension and forced swimming tests (TST & FST) were performed in both groups to establish baseline data for the non-chronic stress condition (Wu *et al*., 2021; Ueno *et al*., 2022). During the experimental period, the sucrose preference test (SPT) was used to sucrose level consumptions (Wu *et al*., 2021). Animals were tested again with TST and FST to confirm the chronic stress inducing depression-like behaviors. Animal Ethical approval for the study was obtained from the Animal Ethics Committee of Khon Kaen University (code: IACUC-KKU [C]- 56/67).

Histopathology and morphometric studies

H&E staining: The head epididymis was fixed with 10 % formalin solution dehydrated by ascending ethanol series before clearing by xylene; it was then infiltrated and embedded with paraffin before sectioning at 5 μm thickness using a semi-automatic rotary. To evaluate histological changes, the paraffin sections in both groups were stained by hematoxylin and counterstained with eosin before observing under light microscope.

Morphometric measurements: The epithelium high and smooth muscle thickness of the epididymis were measured from 10 different serial fields. Each epithelial height was measured from the basement membrane to the apical epithelial surface while smooth muscle thickness of the epididymal duct was measured from the outer margin of the external longitudinal muscular layer to the inner margin of the internal circular muscular layer as explained in Taoto *et al*. (2024).

Masson's trichrome staining: To examine collagen thickness, the tissue section was incubated with Bouin's solution before applying the Trichrome Stain Kit (Abcam, UK). Then, the sections were stained with Weigert's iron hematoxylin (solution A; 1 g of hematoxylin and stained with Masson's trichrome staining kits (Sigma-Aldrich, Inc., USA). The tissue section was incubated with biebrich scarlet-acid fuchsin solution to stain collagens and were stained with aniline blue solution. The accumulated collagens were observed and photographed by Nikon light ECLIPSE E200 microscope equipped with a DXM1200 digital camera.

Western blot analysis.

Left caudal epididymis tissue without sperm mass was extracted with lysis solution. The homogenized tissue was centrifuged at 15,000 rpm to separate the pellet from supernatant. Total protein concentration was measured at a wavelength of 280 nm by using NANO drop spectrophotometer (NanoDrop ND-1000 Spectrophotometer V3.5, Nano Drop Technologies Inc., USA). To investigate the expressions of apoptotic proteins, the total protein sample (120 μg/lane) was separated on 12 % separating gel (SDS PAGE) and transferred onto the nitrocellulose membranes.

Protein membrane was incubated with 5 % BSA for 1 hour in blocking non-specific bonding proteins. The membranes were incubated with primary antibodies including androgen receptor [AR, 1:3000 in 0.1 % TBST (Cat. No 60-680, Merck)], heat shock protein-70 (Hsp-70, 1:5000 [Cat. No MAB3516, Merck]), caspase 3 (1:2000 [Cat. No sc-7272, Santa Cruz Biotechnology]), and caspase 9 (1:2000 [Cat. No sc-56076, Santa Cruz Biotechnology]) for overnight. Then, the individual membrane was washed and further incubated with a specific secondary antibody conjugated with horseradish peroxidase (HRP) including goat anti-rabbit IgG (for anti-AR, Cat. No. AP132P, Merck), or goat anti-mouse IgG (for anti-Hsp-70, caspase 3, and caspase 9 [Cat. No. AP160P, Merck]). The enhanced chemiluminescence (ECL) substrate kit (GE Healthcare Life Science, USA) was used to detect the Ab-Ag complex and visualized under Gel Documentation 4 (Image Quant 600 GE Healthcare, USA). The relative intensity of protein expressions was quantified by using the ImageJ program (Version 1.50i). The GAPDH was used as an internal control to confirm the equal total protein concentration.

Malondialdehyde (MDA) analysis

To measure MDA level, the extracted epididymal tissue was mixed and incubated with 10 % trichloroacetic acid solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 10 minutes. The mixture was added thiobarbituric acid solution and shaken in a water bath at 95 °C for 30 min. After cooling down, the mixture was centrifuged at 10,000 rpm for 5 min. The pink supernatant was measured for its absorbance at 532 nm. A standard curve of standard solution ranging from 0 to 3 nmol/mL was constructed with an R^2 closed to 1 and used for reading MDA levels. The data were expressed as ng MDA/mg of the extracted epididymal protein.

Statistical analysis

All data was expressed as mean ± standard deviation (SD). The difference of means between groups were performed using Mann-Whitney U test (for non-normally distributed data) or independent t-test (for normally distributed data) by GraphPad Prism 8 (GraphPad Software, USA). If the p-value is lesser than 0.05, it was considered as a significant difference between groups.

RESULTS

Effect of DEXA on depressive-like behaviors. The results showed that the immobility times of forced swimming and tail suspension significantly were increased in the DEXA group compared to the control. It was found that the percentage of sucrose preference of DEXA group seemed to be lower than that of control, but it was not statistically different as shown in Figure 1.

Effect of DEXA on gross morphology, body weight, and epididymal weight. Compared to the control group, it was observed that the morphology of epididymis plus vas deference of DEXA rats is slightly smaller (Fig. 2A). As shown in Figure 2B, the final body weight of DEXA group was significantly lower than the control group. Significantly, both absolute and relative weights of the epididymis plus vas deference were greater than those of control ($p < 0.05$, Figs. 2C, D).

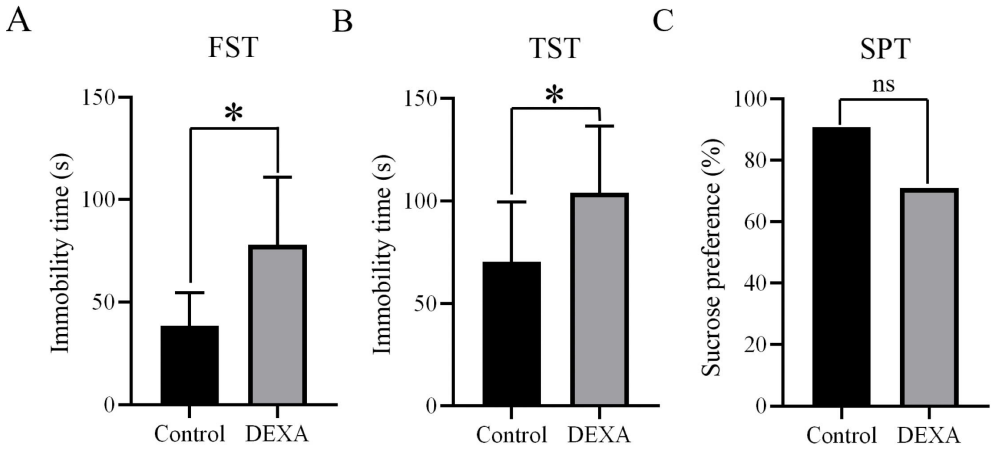


Fig. 1.The chronic stress responses determined by depression-like behavior tests compared between control and dexamethasone (DEXA) groups. A) FST, forced swimming test; B) TST, tail suspension test; C) SPT, sucrose preference test *$p < 0.05$, statistically significant difference compared between groups. ns: no significant difference.

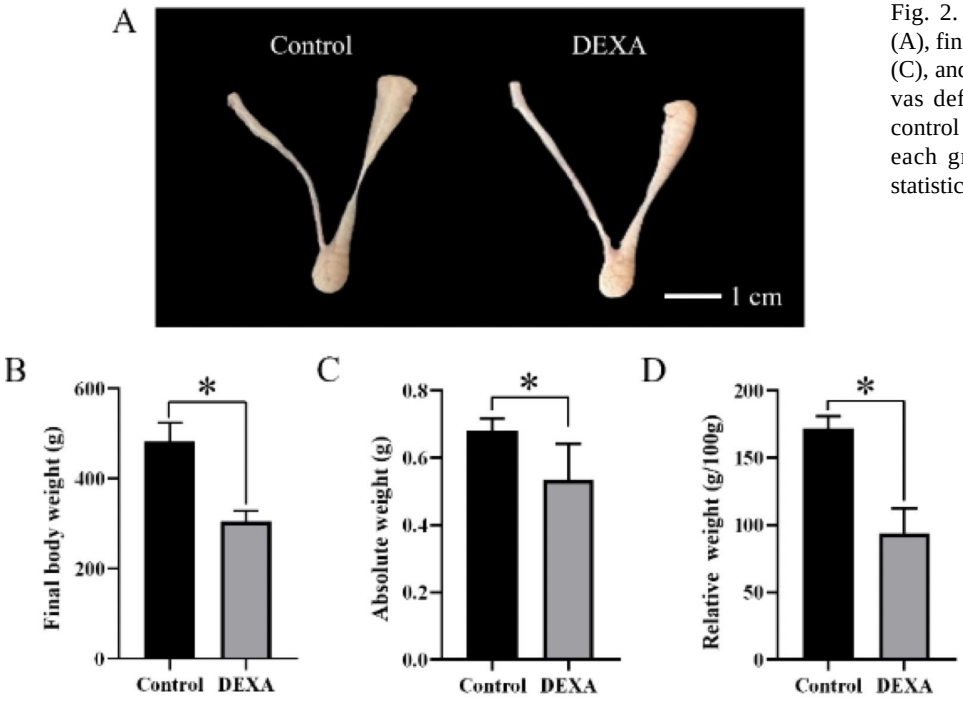
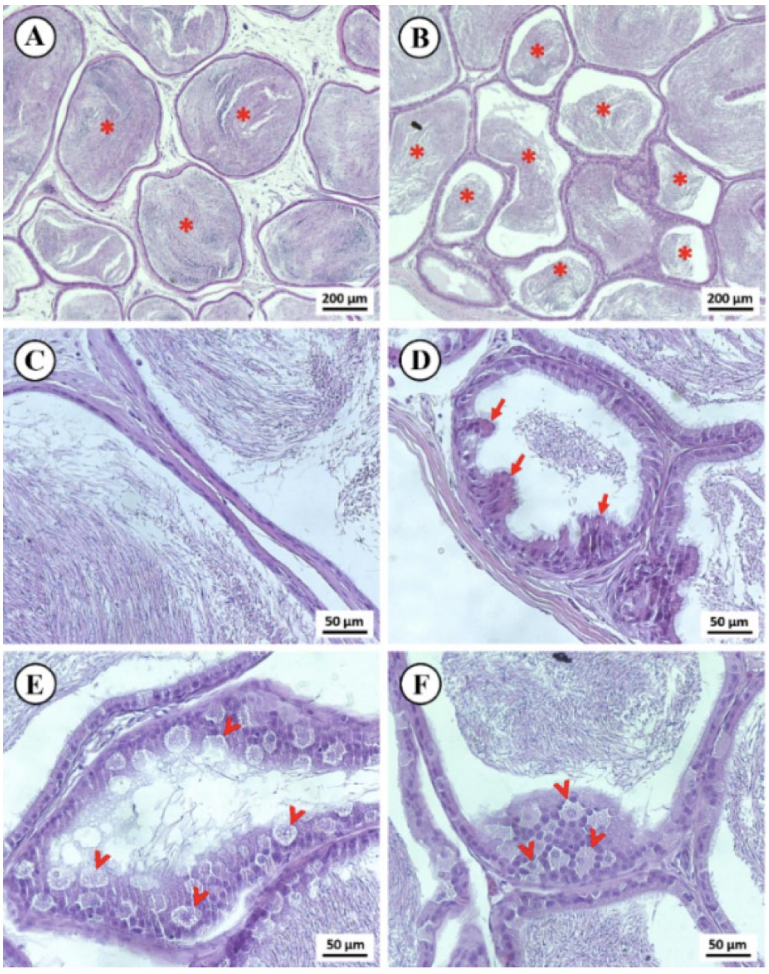


Fig. 2. Representative gross morphology (A), final body weight (B), absolute weight (C), and relative weight of epididymis plus vas deference (D) as compared between control and DEXA treated groups (n=10, each group). The asterisk (*) indicates statistical significance at $p < 0.05$.



Effect of DEXA on epididymal sperm mass and histology of rat cauda epididymis. The histological features of caudal epididymis in both groups were shown in Figure 3. It was observed that the sperm mass of the DEXA group (Fig. 3B) was obviously decreased when compared to the control group (Fig. 3A). In addition, the hyperplasia of clear cells and cribriform change were found only in the DEXA epididymal epithelium (Figs. 3D-F) as compared to the control (Fig. 3C).

Fig. 3. Micrographs showing the sperm mass within the cauda epididymis lumen (A, B) and histology of cauda epididymal epithelium (C-F) compared between control and DEXA treated groups. Red asterisks; epididymal sperm mass, arrows; cribriform change, and arrowheads; hyperplasia of clear cells.

Effect of DEXA on morphometrics of cauda epididymal. The epithelial height of DEXA epididymis was significantly increased compared to the control (Figs. 4A, B). Interestingly, the smooth muscle thickness of cauda epididymal epithelium was significantly thicker than that of control group (Fig. 4 E). Compared between groups, it was found that the percentage of collagen fiber area was not statistically different as shown in Figures 4C-D, F ($p<0.05$).

DEXA increased the malondialdehyde level in cauda epididymal tissue. It was demonstrated that the level of malondialdehyde (MDA) level in the cauda epididymal tissue of DEXA group was significantly increased as compared to control (Fig. 5).

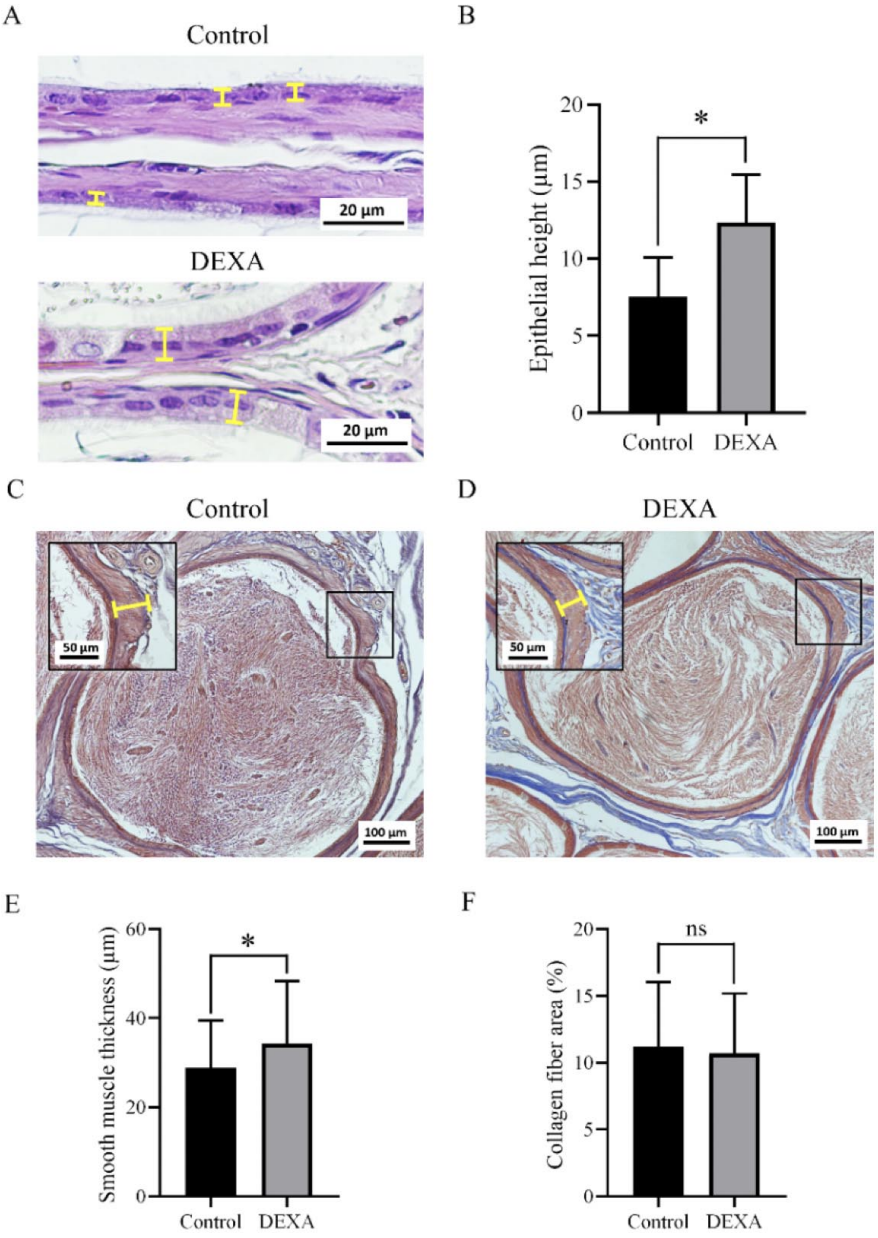


Fig. 4 . Cauda epididymis sections stained with H&E and Masson's trichrome. Measurements of epididymal epithelial height (A, B), smooth muscle thickness (C-E) and the percentage of collagen fiber area (F). *$p < 0.05$, statistically significant difference as compared between groups. ns: no significant difference.

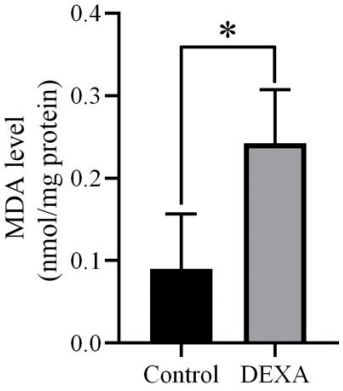


Fig. 5. Comparison of malondialdehyde (MDA) level in the epididymal tissue of control and DEXA-treated groups for 21 consecutive days. *$p < 0.05$, statistically significant difference.

Effect of DEXA on expressions of caspases, Hsp-70, and androgen receptor. The results showed that expression of pro-caspase 3 was significantly decreased in DEXA epididymis, but that of pro-caspase 9 was not significantly different as compared to control (Figs. 6A, B). In addition, the Hsp-70 and AR expressions were not significantly different between groups although the intensity of AR expression in DEXA group tended to be lower than that of control (Figs. 6C, D).

DISCUSSION

Epididymis is known to be an essential male reproductive organ that is the place for not only for storage but also for functional maturation of testicular sperm. Therefore, the changes of ductus epididymis epithelial cells and its substances in the epididymal fluid will affect sperm physiological functions like sperm motility, capacitation, and acrosome reaction, respectively. It has been demonstrated that drugs and other conditions such as diabetes and CS can cause structural and biochemical alterations in the

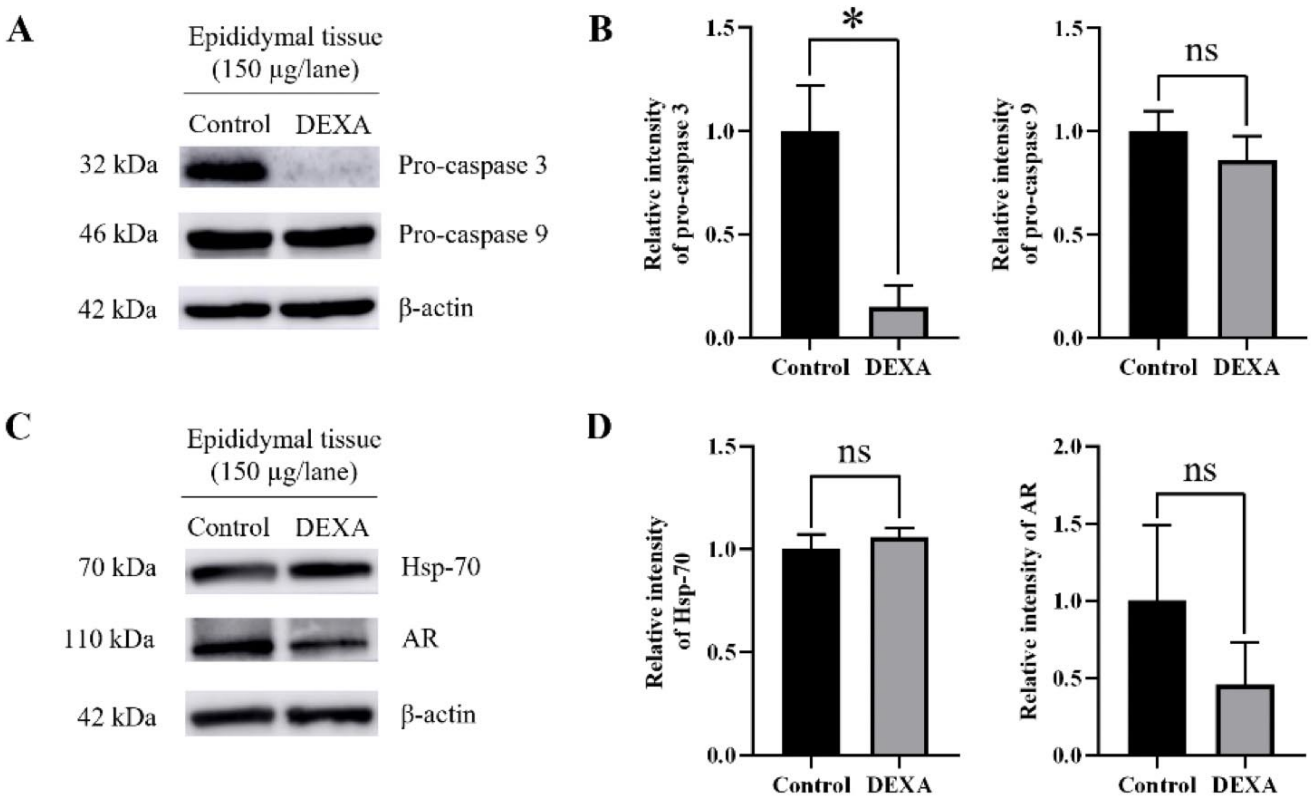


Fig. 6. Expressions with their relative intensities of pro-caspases 3 and 9 (A-B), heat shock protein-70 (Hsp-70), and androgen receptor (AR) in the cauda epididymal tissue lysates (C and D). b-actin was used as an internal control. The asterisk (*) indicates the statistical significance at $p<0.05$. ns; no significant difference.

epididymis. Previously, valproic acid was shown to change the expressions of tyrosine phosphorylated proteins in rat epididymis (Iamsaard *et al*., 2017; Sawatpanich *et al*., 2018). In addition, ethyl alcohol caused histopathology of the epididymis with alteration in the luminal metabolite composition (Taoto *et al*., 2024). Indeed, the increased MDA levels and changes of tyrosine phosphorylation and histology in epididymal secretion and tissue have been documented in CS animal models induced with physical stressors (Guo *et al*., 2020; Arun *et al*., 2021; Choowong-In *et al*., 2021a,b; Arun *et al*., 2022; Lapyuneyong *et al*., 2022). Although the unpredictable stressors were previously used to mimic the CS in experimental animals, its weak points are complicated handling, time consuming, and half successful induction. Alternatively, DEXA tends to be commonly used for CS with the depressive-like behaviors. Many studies reported that DEXA decreased the epididymis weight with decreased sperm quality (Silva *et al*., 2014; Jeje *et al*., 2017; Kempinas *et al*., 2019b; Jeje *et al*., 2020; Nassan *et al*., 2021; Azimi Zangabad *et al*., 2023). This study demonstrated the lower sperm mass and epithelial height in DEXA with slight fibrosis in the ductus wall, indicating some dysfunctions of the epididymis. Pro-caspase 3 expression increased in this study was assumed to be increased in its mature form of caspase 3 involved in apoptotic pathway. Similarly, the increased MDA and caspase 3 in epididymis found in this study may be same as DEXA mechanism, as previously reported in mouse and hamster testes (Mukherjee *et al*., 2015; Sadeghzadeh *et al*., 2019). Taken together, the morphohistometry changes of cauda epididymis with some apoptotic marker expressions that were increased in this study can be used to explain the decrease of semen volume and sperm quality as reported in previous studies (Gür *et al*., 2005; Ozer Kaya *et al*., 2020)

CONCLUSION

It was concluded that DEXA induced chronic stress with depressive-like behavior and caused histopathology in cauda epididymis through lipid oxidation of MDA and caspase 3 pathways. This new knowledge is additional information to support using of DEXA as a potential chronic stress induction for studying functional immaturity of epididymal sperm in depressive male in the near future.

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RESUMEN: El estrés crónico (EC) con un aumento del nivel de cortisol se considera un factor de riesgo que promueve comportamientos similares a la depresión y puede causar numerosos trastornos, incluida la infertilidad masculina. La dexametasona (DEXA), un corticosteroide sintético, no solo se utiliza para tratar la inflamación, sino también para inducir el síndrome de choque térmico (SC) en modelos animales. Si bien se ha demostrado que la DEXA daña los parámetros reproductivos masculinos, nunca se han reportado sus efectos adversos en el epidídimo, importante para la maduración espermática. Este estudio tuvo como objetivo investigar las alteraciones histológicas y la expresión proteica en el epidídimo inducido con DEXA. Veinte ratas macho adultas se dividieron en grupos control y DEXA (1,5 mg/kg de peso corporal) mediante tratamiento durante 21 días consecutivos (10 ratas/grupo). El estrés crónico con comportamientos depresivos se determinó mediante pruebas de natación forzada y suspensión de la cola. Se examinaron la histopatología, el nivel de malondialdehído (MDA) y la expresión de pro-caspasas (3, 9), el receptor de andrógenos (AR) y la proteína de choque térmico-70 (Hsp-70) en el epidídimo. Los resultados mostraron que el peso y la masa espermática del epidídimo caudal se redujeron en ratas DEXA con algún cambio cribiforme e hiperplasia de células claras. La DEXA aumentó significativamente la altura de las células epiteliales del epidídimo, el grosor de la pared muscular y los niveles de MDA, pero no se observaron diferencias en el área de las fibras de colágeno en comparación con el control. La expresión de procaspasa 3 disminuyó significativamente y el AR tendió a una baja expresión en el tejido epididimario sometido a DEXA. No se observaron diferencias en la expresión de pro-caspasa 9 y Hsp-70 entre los grupos. En conclusión, la DEXA indujo la histopatología de la cola del epidídimo mediante la oxidación lipídica y la activación de la caspasa 3. Esto podría utilizarse para explicar la inmadurez funcional de los espermatozoides epididimarios en hombres con síndrome de Guillain-Barré con comportamiento depresivo.

PALABRAS CLAVE: Estrés crónico; Dexametasona; Cauda epidídimo; Malondialdehído; Caspasa 3.

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