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Influence the Content of Steroid Hormones and Expression of Steroidogenic Enzymes with DHEA-Treated in TM-3 Cells

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Abstract: The adrenals of humans and primates could secrete large amounts of Dehydroepiandrosterone (DHEA) and its sulphate ester (DHEA-S) in the circulation which act as precursors of active steroid hormones in a long series of peripheral target intracrine tissues. The marked decline of serum DHEA and DHEA-S concentrations with age in humans has been incriminated in the development of various pathologies. Therefore, this study aims to provide detailed information on the effects of DHEA on steroid hormones and its metabolites by Liquid Chromatography-tandem Mass Spectrometry (LC-MS) in TM-3 cells, a Leydig cell line. The results showed that the concentration of DHEA rapidly declined post-treatment while testosterone and estradiol content increased over the same time-period in TM-3 cells following treatment with 100 μM DHEA. Concomitantly, the concentration of progesterone slightly increased and cortisol content decreased over the treatment period in TM-3 cell. Furthermore, DHEA treatment produced a significant increased in the 3β-Hydroxysteroid Dehydrogenase (3β-HSD) and 17β-Hydroxysteroid Dehydrogenase (17β-HSD) protein expression following incubation with DHEA for 24-48 h but the protein expression of aromatase decreased in the presence of DHEA for 24-48 h. The results of present study indicate that DHEA could transformed into steroid hormones by regulated expression of steroidogenic enzymes in TM-3 cells.

Key words: DHEA, steroid hormones, steroidogenic enzymes, TM-3 cell, cortisol

INTRODUCTION

Dehydroepiandrosterone (DHEA), the most abundant steroid hormone in the circulation is a major secretory product of the adrenal gland (Kroboth *et al*., 2003). Both DHEA and its sulphate ester (DHEA-S) exhibit a characteristic age related pattern of secretion. DHEA secretion in humans reaches its maximum level between the second and third decades of life then it begins a steady decline at a rate of 2% per year until only 10% the maximal level remains at age 70 or 80 (Genazzani *et al*., 2007). Earlier research has shown that DHEA exerts numerous beneficial effects including the prevention of obesity (Sato *et al*., 2012), hypoglycemia (Ping *et al*., 2000), cancer (Arnold *et al*., 2008), atherosclerosis (Savineau *et al*., 2013) and age-induced changes to the brain (Majewska, 1995). DHEA exerts its action either indirectly in the peripheral target tissues of the sex steroid hormones (following conversion to the androgens, estrogens or both) or directly as a neurosteroid (by interacting with neurotransmitter receptors in the brain). Analysis of the pharmacokinetics of DHEA and DHEA-S following exogenous administration of DHEA, reveals that both forms of the hormone undergo continuous inter-conversion (Arlt *et al*., 1999). Whereas, DHEA-S is the hydrophilic storage form that circulates in the blood, only lipophilic DHEA can be converted into the androgens and estrogens. As a steroid hormone precursor, DHEA can be converted into androstenedione by 3β-Hydroxysteroid Dehydrogenase (3β-HSD) in peripheral target tissues and then undergo further conversion to testosterone and estradiol by 17β-Hydroxysteroid Dehydrogenase (17β-HSD) and aromatase, respectively (Labrie *et al*., 2001). Labrie *et al*. (2007) showed that serum levels of androstenedione, testosterone and dihydrotestosterone were significantly increased in 55-65 years old post-menopausal women following twice daily percutaneous application of a DHEA emulsion. Song *et al*. (2010) demonstrated that serum concentrations of testosterone, estradiol, cortisol and aldosterone markedly increased in rat testis following treatment with DHEA. When DHEA was administered orally, intravenously (i.v.) and during a continuous i.v., infusion in ovariectomized cynomolgus monkeys, the major circulating DHEA metabolites that appeared were DHEA-S, androsterone glucuronide and androstane-3-

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alpha while levels of testosterone, dihydrotestosterone and androstenedione were minimal and no transformation to estrogens could be detected (Leblanc *et al*., 2003). This may indicate that the circulating hormone concentration does not completely reflect the conversion of DHEA in the peripheral target tissues or cells (Arlt *et al*., 1999). Leydig cells produce 90-95% of the testosterone in the circulation and express various steroidogenic and metabolic enzymes (Haider, 2004). As peripheral target cells, the metabolism of DHEA in Leydig cells is still unclear.

Since, as mentioned earlier, DHEA is believed to play an important role against various ailments, it is important to investigate the metabolism of DHEA in target tissues and cells. The previous study had verified that DHEA can effect the cell growth and mitochondrial function in TM-3 cells (Shen *et al*., 2012). Therefore, this study was carried out to further investigate the affect of DHEA on the steroid hormones and its metabolites content by Liquid Chromatography-tandem Mass Spectrometry (LC-MS) and the protein expression of steroidogenic enzymes in TM-3 cells. This information is necessary to fully understand the mechanism of DHEA exerts its biological effects *in vivo*.

MATERIALS AND METHODS

TM-3 cells, a specifically peripheral target cell which is a Leydig cell line and isolate from testis of immature BALB/c mice were obtained from the American Type Culture Collection (Rockville, USA).

DHEA and Dimethyl Sulfoxide (DMSO) were purchased from Sigma (Sigma, USA), Trypsin obtained from the Sunshine Biotech Co. (Nanjing, China), the protein assay kits were procured from the Beyotime Institute of Biotechnology (Wuhan, China). The testosterone Radioimmunoassay (RIA) kits were purchased from the Beijing. Beifang Biotechnology Institution (Beijing, China). Cortisol, DHEA, testosterone, estrone, estradiol, progesterone, corticosterone and 17α-hydroxyprogesterone were obtained from Sigma (Louis, USA).

Cell culture: TM-3 cells were seeded in 6 well plastic culture plates with a density of 1×10^6 cells/well in DMEM-F12 (1:1) medium. Supplements were added using 5% equine serum (Hyclone, USA), 2.5 mM L-glutamine, 1.0 mM hydroxyethyl piperazine ethanesulfonic acid and 2.5% fetal bovine serum (Hyclone, USA). The culture medium also contains streptomycin (100 μg mL^{-1}) and penicillin (100 IU mL^{-1}). TM-3 cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. DHEA was dissolved in DMSO in the 10 mL tube and the final concentration of DMSO was 0.1%. It was then diluted with medium before being used in the experiments.

Detection of testosterone content by Radioimmunoassay (RIA): The concentration of testosterone in TM-3 cells under basal or stimulated conditions was determined using an RIA kit. After culturing for 48 h in DMEM-F12 medium at 37°C, cells were incubated with a dimethyl sulfoxide solution of DHEA (0, 0.1, 1, 10 or 100 μM) for 24 h or with 100 μM-DHEA for various lengths of time (0, 3, 12, 24 or 48 h) in DMEM-F12 medium at 37°C (n = 6). Following incubation, cells were harvested and then disrupted ultrasonically in ice, centrifuged at 2500×g for 10 min at 4°C and the supernatants were then collected and stored for subsequent analysis at -20°C. The concentrations of testosterone in the supernatants were determined according to the manufacturer's instructions.

Detected of steroid hormones by Liquid Chromatography-tandem Mass Spectrometry (LC-MS): The cell cultured as 2.3 (n = 6). Cells were incubated with a dimethyl sulfoxide solution of 100 μM DHEA for 0, 3, 12, 24 or 48 h. Following incubation, cells were harvested and then disrupted ultrasonically in ice, centrifuged at 2500×g for 10 min at 4°C. The 5 mL of the supernatants were collected and freeze-dried and reconstituted with 300 μL of distilled water, the samples were then stored at -20°C for subsequent analysis. The 20 μL of each sample were combined with 100 μL of acetonitrile and shaken with a suspension instrument and the upper organic layer was collected for analysis. Standard samples for cortisol, dehydroepandrosterone, estradiol, estrone, progesterone, testosterone, corticosterone and 17α-hydroxyprogesterone were dissolved with acetonitrile and used to prepare calibration standards, the final concentration of standards were 5.0, 12.5, 25 and 125 ng mL^{-1}. These were stored at -20°C for subsequent analysis. A Dionex Ultimate 3000 (Thermo, USA) separation module, equipped with a quaternary pump and a column oven was used for chromatographic separation. Separation was achieved at 30°C on a Gemini AAAC18 column (4.6×150 mm, 5 μm). The injection volume was 20 μL and the flow rate was 800 μL min^{-1}. The gradient used for the elution of samples is described in Table 1.

Table 1: Sample elution gradients

	Mobile phase	
Time (min)	A (water) (%)	B (acetonitrile) (%)
0.00	50	50
0.50	5	95
7.5	0	100
12.0	50	50
15.0	50	50

All analyses were performed on an ABI-3200 Q TRAP triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) and equipped with a direct online inlet system and an atmospheric pressure chemical ionization heated nebulizer interface was operated in the positive mode with a nebulizer current of 3000 V. The source temperature was adjusted to 550°C and the entrance potential was set at 10 V. Nitrogen was used as the nebulizer gas (40 psi) and collision gas (medium). The ion spray voltage was set at 5500 V, the source temperature was 450°C and the nitrogen pressure (turbo ion gas) was 70 psi. The multiple reaction monitoring was used to measure analytes by monitoring at least two transitions for each substance with dwell times between 100 and 200 msec. The resolution of quadrupole 1 and 3 was set to unit (Schmidt *et al*., 2009).

Total protein extract and western blotting: The cells were cultured and treated as section 2.3 (n = 6). Cells were collected and protein was extracted for the subsequent determination of 3β-HSD, 17β-HSD and aromatase levels. Total protein extracts were prepared by using commercial kit (Biosynthesis Biotechnology Co., Beijing, China). The protein concentration was measured using a bicinchoninic acid protein determination kit.

A quantity of 60 μg cells extract protein was separated on 10% SDS-polyacrylamide denaturing gel. Following SDS-PAGE, protein electrophoretically transferred to ImmobiloneTM-P polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane were then blocked at 25°C for 1 h in blocking buffer (2-amino-2-hydroxymethyl-propane-1, 3-diol (Tris)-buffered saline (TBS: pH 7.6; Tris base 2.42 g L^{-1}, NaCl 8 g L^{-1}), 0.1% Tween 20, 5% non fat dry milk). Proteins were then incubated at 4°C overnight with rabbit anti-3β-HSD antibodies (1:1000 dilution; Abcam, USA), rabbit anti-17β-HSD antibodies (1:1000 dilution; Santa Cruz, CA), rabbit anti-aromatase (1:1000 dilution; Abcam, USA) and rabbit anti-GAPDH (1:1000 dilution; CST, USA). After washing with TBS (pH 8.0) containing 0.1% Tween-20, goat anti-rabbit IgG with horseradish peroxidase-conjugated (1:5000, Bioworld Technology Co., USA) in washing solution was added and incubated for 1 h at room temperature.

The immunoreactive proteins were detected by superSignal chemiluminescence. The protein bands were digitally imaged for densitometric quantification with a software program (Eastman Kodak Company, Rochester, NY, USA). The protein expressions were normalized to Glyceraldehyde-3-Phosphatedehydrogenase (GAPDH).

Statistical analysis: The results were expressed as the mean±SE and differences were considered significant when $p<0.05$. Data were analyzed with Student's t-test for independent samples or one-way Analysis of Variance (ANOVA) (*$p<0.05$, **$p<0.01$) and correlation analysis was performed using Statistical Software SPSS13.0 (StateSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Effect of DHEA on the content of testosterone in TM-3 cells

Dose-dependent effect of DHEA on the content testosterone in TM-3 cells: In the present study, researchers first determined the effect of DHEA on the testosterone content in TM-3 cells using RIA. Cells were incubated with a dimethyl sulfoxide solution containing DHEA (0.1-100 μM) or vehicle for 24 h at 37°C. As described in Fig. 1a, testosterone content was not detected in the control group while a marked increase in testosterone content was evident in the 0.1-100 μM DHEA-treated groups. The concentration of testosterone was dose-dependent and the DHEA concentration of 100 μM produced the maximum content of testosterone (16.65 ng mL^{-1}) (Fig. 1a).

Time-dependent effect of DHEA on the content testosterone in TM-3 cells: Thus, researchers subsequently used 100 μM DHEA to culture TM-3 cells for the various time periods at 37°C. As illustrated in Fig. 1b, the testosterone content was significantly higher during the experimental period from 3-48 h as compared to the control group. The testosterone content increased by 5965.91% from 3-4 h (from 0.663±0.047 to 40.217±1.142 ng mL^{-1}) (Fig. 1b).

The effect of DHEA on the content of steroid hormones in TM-3 cells

The specificity, precision and recovery of Liquid Chromatography-tandem Mass Spectrometry (LC-MS): The mass spectrometer ionization source settings were optimized for maximum precursor ion yields for each steroid and the parameters are summarized in Table 2. Two mass transitions were optimized for each analyte with a single transition being used to monitor the corresponding deuterated internal standards. The first transition was used to quantify the target analyte and the second was used to qualify the identity of the target compound using a confirmatory ion ratio. The peak area ratios of each substance were correlated with the standard concentration over the range of 5-500 ng mL^{-1}. The regression coefficient for all analytes was >0.951 over

Table 2: Ion transitions and respective mass spectrometric parameters for the determination of trenbolone and analogous substances. Italics denote qualifier ions

Analytes	Retention time (min)	Ione transition (m/z)	Declustering potential (V)	Collision energy (mV)	Dwell time (msec)
Cortisol	2.69	363-121	30	26	150
Cortisol	2.70	363-91	30	54	150
Dehydroepiandrosterone	3.68	289-271	17	9	200
Dehydroepiandrosterone	3.76	289-253	17	9	200
Estradiol	3.60	255-133	25	19	150
Estradiol	3.36	255-159	25	20	150
Estrone	3.78	271-159	25	20	200
Estrone	3.77	271-157	25	20	200
Progesterone	4.38	315-97	30	25	100
Progesterone	4.50	315-109	30	25	100
Testosterone	3.68	289-97	33	26	100
Testosterone	3.68	289-109	33	26	100
Corticosterone	3.09	347-121	60	35	150
Corticosterone	3.09	347-108	60	35	150
17α-hydroxyprogesterone	3.72	331-109	80	37	150
17α-hydroxyprogesterone	3.72	347-121	60	35	150

Table 3: Concentration ranges, calculated calibration functions and correlation coefficients

Compounds	Concentration range (ng mL^{-1})	Calibration function ($Y = b_1X+b_0$)	r^2
Cortisol	5-500	Y = 181X+453	0.9980
Dehydroepandrosterone	5-500	Y = 54.5X+411	0.9970
Estradiol	5-500	Y = 10.8X+226	0.9951
Estrone	5-500	Y = 3.71X-22.2	0.9511
Progesterone	5-500	$Y = 2.21e^{+003}X-3.34^{e+003}$	0.9815
Testosterone	5-500	$Y = 1.76^{e+003}X+98.4$	0.9988
Corticosterone	5-500	Y = 193X-997	0.9963
17α-hydroxyprogesterone	5-500	Y = 744X-989	0.9992

their clinical concentration ranges (Table 3) and all calculated concentrations for the calibrators were within ±10% of the assigned values (Table 4). The inter-assay CV and deviation from assigned values was ≤10.46% for all analytes. The mean recovery for each QC taking into account the internal standard was from 82.4-111.21% for all steroids (Table 4).

The content of steroid hormones: As described in Table 5 after 0, 3, 12, 24 and 48 h treatments with 100 μM DHEA, the concentration of DHEA decreased from 32.26 ng mL^{-1} (0 h) to 17.90 ng mL^{-1} (48 h) with the lowest level of DHEA (13.48 ng mL^{-1}) evident at 24 h. The concentration of testosterone increased following treatment with DHEA with a maximum concentration of testosterone occurring at 48 h (5.41 ng mL^{-1} or 20.82 times that at 3 h (0.26 ng mL^{-1}) while the testosterone concentration was below the detection limit at 0 h.

Incubation with DHEA increased the concentration of estradiol with a maximum concentration occurring at 48 h (167.16 ng mL^{-1}) which was 10.95 times that at 0 h (15.26 ng mL^{-1}). The concentration of cortisol, although minimal in all samples, decreased from 2.764 ng mL^{-1} at 0 h to 0.049 ng mL^{-1} at 48 h.

The concentration of estrone (a weak estrogen) in the samples decreased from 2801 ng mL^{-1} at 0 h to 1535 ng mL^{-1} at 48 h and the lowest value (1118 ng mL^{-1}) appeared at 24 h. Progesterone was not detected 0-3 h after DHEA treatment while it increased slightly from 12-48 h. Neither corticosterone nor 17α-hydroxyprogesterone was detected in the samples during the assay period.

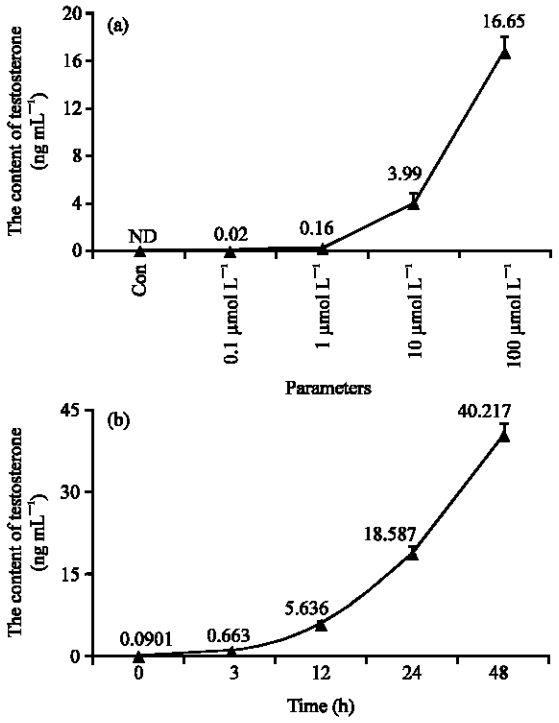

Fig. 1: Effect of DHEA on the content of testosterone; TM-3 cells were incubated with various concentrations of DHEA (0.1-100 μM) or vehicle (0 μM) for 24 h; a) or with 100 μM-DHEA for various periods of time; b) at 37°C. The concentration of testosterone in TM-3 cells was determined using a RIA kit. Results showed that a dose-dependent manner increase in testosterone was found in 0.1-100 μM DHEA-treated groups and the content of testosterone was significantly increased during the experimental period from 3-48 h. Values are means (n = 6); ND: Not Detected

Correlation analysis of steroids: To study the mechanism of DHEA biotransformation, oscillations in the levels of various steroids were monitored during DHEA incubation and correlations among the different steroids were showed in Table 6. The results showed that DHEA was negatively correlated ($p<0.05$) with testosterone and estradiol.

However, DHEA was positively correlated ($p<0.01$) with estrone and cortisol. Incubation of cells with 100 uM DHEA resulted in an increased concentration of testosterone and estradiol over the 48 h incubation periods while the concentrations of DHEA, estrone and cortisol gradually decreased. Similarly, testosterone was positively correlated ($p<0.01$) with estradiol and progesterone but negatively correlated ($p<0.05$) with estrone and cortisol. As well, estradiol was positively correlated ($p<0.01$) with progesterone and negatively correlated ($p<0.01$) with estrone and cortisol while there was a positive correlation between estrone and cortisol.

Effect of DHEA on the protein expression of 3β-HSD, 17β-HSD and aromatase: As shown in Fig. 2 with the extension of culture time, the protein expression levels of 3β-HSD, 17β-HSD and aromatase were not changed in the control group. Compared to the control, 3β-HSD protein expression increased ($p<0.05$) subsequent to DHEA incubation for 24-48 h (Fig. 2a) and the level of 17β-HSD also increased ($p<0.05$) following 48 h of DHEA treatment (Fig. 2b). However, expression of aromatase decreased ($p<0.05$) in the presence of DHEA for 24-48 h (Fig. 2c).

The earlier study had demonstrate that DHEA inhibited TM-3 cell growth whereas it improved TM-3 cell viability by increasing mitochondrial membrane permeability and the activity of succinate dehydrogenase (Shen *et al*., 2012). Thus, this research was undertaken to

Table 4: Linearity of inter-day calibration curves based on mean values, percent Coefficients of Variation (CV%) and accuracy and recovery data evaluated at each QC level

Compounds	Standard concentration (ng mL^{-1})	Detected concentration (ng mL^{-1})	CV (%)	Recovery (%)
Cortisol	5.0	4.92±0.03	0.51	98.40±5.09
	12.5	12.79±0.06	0.47	102.30±4.85
	25.0	25.71±0.15	0.57	102.85±5.83
	125.0	130.00±3.61	2.77	104.00±2.88
Dehydroepandrosterone	5.0	4.99±0.10	2.06	99.76±1.80
	12.5	12.28±0.21	1.74	98.24±1.71
	25.0	26.02±1.50	5.75	104.08±5.99
	125.0	122.00±4.60	3.77	97.60±3.68
Estradiol	5.0	4.96±0.16	3.27	99.12±3.23
	12.5	12.40±0.36	2.93	99.20±2.91
	25.0	26.22±1.18	4.50	104.88±4.72
	125.0	116.80±4.83	4.14	93.44±3.87
Estrone	5.0	5.20±0.35	6.77	104.01±7.04
	12.5	11.96±0.62	5.14	95.68±4.92
	25.0	24.66±1.20	4.87	98.64±4.81
	125.0	134.20±8.13	6.06	107.36±6.51
Progesterone	5.0	5.34±0.24	4.53	106.80±4.83
	12.5	10.30±1.08	10.46	82.40±8.62
	25.0	27.88±2.48	8.90	111.21±9.93
	125.0	126.00±1.41	1.12	100.80±1.13
Testosterone	5.0	5.09±0.14	2.77	102.02±2.83
	12.5	11.70±0.55	4.71	93.61±4.41
	25.0	25.81±1.33	5.14	103.20±5.31
	125.0	123.20±2.48	2.01	98.56±1.98
Corticosterone	5.0	4.88±0.34	7.01	97.56±6.84
	12.5	12.98±0.69	5.35	103.84±5.55
	25.0	24.53±1.07	4.35	98.14±4.26
	125.0	118.18±5.95	5.03	94.56±4.76
17α-hydroxyprogesterone	5.0	5.08±0.34	6.63	101.64±6.74
	12.5	12.34±0.56	4.55	98.72±4.49
	25.0	26.25±0.97	3.69	105.01±3.87
	125.0	124.09±2.58	2.08	99.28±2.06

Values are expressed as mean and standard deviation (n = 6)

Table 5: Concentration of steroids incubated with DHEA

Steroids (ng mL^{-1})	Time (h) 0	3	12	24	48
Cortisol	2.764±0.66	1.118±0.071	0.272±0.036	0.369±0.185	0.049±0.007
Estradiol	15.26±0.60	64.31±7.91	78.12±5.40	95.88±6.40	167.16±15.13
Testosterone	ND	0.256±0.02	0.852±0.099	1.505±0.28	5.412±0.51
Dehydroepiandrosterone	32.26±3.56	34.12±1.33	18.66±2.50	13.48±2.76	17.90±11.76
Estrone	2801±60.13	2783±231.25	1710±102.08	1118±141.52	1535±52.46
Progesterone	ND	ND	12.72±0.76	13.18±0.79	15.04±0.90
Corticosterone	-	-	ND	-	-
17α-hydroxyprogesterone	-	-	ND	-	-

The content of steroids was detected by LC/MS. Results are means±SE (n = 6); ND = None Detected

Table 6: Correlation analysis of steroids

Steroids	Dehydroepiandrosterone	Testosterone	Estradiol	Estrone	Cortisol	Progesterone
Dehydroepiandrosterone	1.000					
Testosterone	-0.515*	1.000				
Estradiol	-0.639*	0.927**	1.000			
Estrone	0.958**	-0.543*	-0.682**	1.000		
Cortisol	0.752**	-0.547*	-0.761**	0.723**	1.000	
Progesterone	0.028	0.945**	0.960**	0.031	-0.682*	1

A positive value present negative correlation, a value negative present positive correlation; *p<0.05; **p<0.01

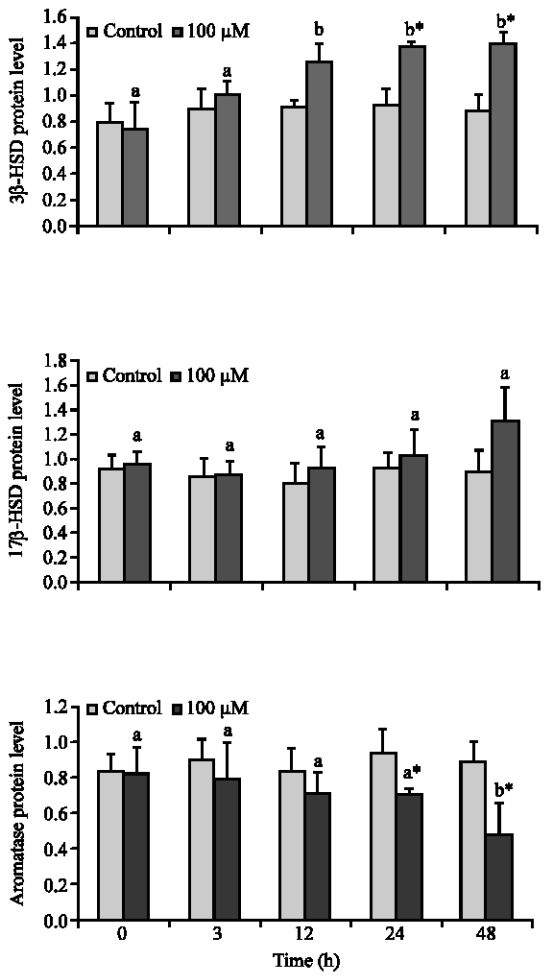

Fig. 2: Effect of DHEA on 3β-HSD, 17β-HSD and aromatase protein expression. Results of statistical analysis for protein expression were normalized to GAPDH; a) 3β-HSD; b) 17β-HSD and c) aromatase. Values are means±SE (n = 6). The different letters indicates significant differences in different periods in DHEA-treated groups, $p<0.05$. Mean value was significantly different from that the treated groups compared with control group, respectively, *$p<0.05$

study the effect of DHEA on the steroid hormones content and expression of enzymes which are involved in active steroid hormone biosynthesis using TM-3 cells treatment with 100 μM DHEA over a 48 h time-period. The results indicate that treatment with DHEA leads to a higher concentration of testosterone in TM-3 cells in a dose and time-dependent fashion (Fig. 1). These data are consistent with the earlier study that intraperitoneal injection of DHEA can lead to high serum levels of testosterone in the male rats (Song *et al*., 2010). Hence, these data may well suggest that DHEA treatment significantly increases the content of testosterone in TM-3 cells and that this provides a theoretical basis for the further study of the effect of DHEA on the steroid hormones content and the expression of steroidogenic enzymes.

In the present study, it was observed that the DHEA content increased at 3 h, decreased at 12 h and increased again at 48 h in TM-3 cell. Aoki *et al*. (2004) reported that serum DHEA-S in human plasma is approximately 300 fold higher than that of DHEA and 20 fold higher than that of any other steroid hormone. Analysis of the pharmacokinetics of DHEA and DHEA-S following exogenous administration of DHEA reveals that DHEA and DHEA-S undergo continuous inter-conversion and DHEA-S conversion from DHEA depends on a ubiquitous sulphatase enzyme also DHEA-S is likely converted back to DHEA on a continuous basis by sulphatase to act as a DHEA reservoir resulting in the delivery of active androgens to the peripheral target tissues (Arlt *et al*., 1999). The results showed that the DHEA content decreased from 3-48 h while testosterone content increased over the same time-period in TM-3 cells (Table 5). Also, correlation analysis results showed that there was a significant negative correlation between DHEA and testosterone (Table 6). The results concur with those of Leblanc *et al*. (2002) who reported that following administration of DHEA to cynomolgus monkeys, serum concentrations of androstenedione and testosterone were significantly increased. As a steroid hormone precursor, DHEA can be transferred into active androgens and/or estrogens depends upon the expression levels of the various steroidogenic enzymes in the peripheral tissues (Labrie *et al*., 2001). In the present study, the expression level of 3β-HSD protein increases following incubation with DHEA for 24-48 h (Fig. 2a) and that the 17β-HSD protein level also increases following DHEA treatment for 48 h (Fig. 2b). The data indicate that DHEA can be converted to testosterone in TM-3 cells and that enzyme expression levels of 3β-HSD and 17β-HSD are directly related to steroid biosynthesis in this process. The pharmacokinetics of DHEA differ between men and women and DHEA-induced increase in circulating estrogens may contribute to beneficial effects of DHEA in men (Frye *et al*., 2000; Aoki *et al*., 2004). In the current study, the estradiol content increased significantly while the estrone content decreased during the same interval (Table 5). Correlation analysis results showed that DHEA was negatively correlated with estradiol and positively

correlated with estrone while estradiol was negatively correlated with estrone (Table 6). Song *et al.* (2010) also reported that serum estradiol levels increased in treatment of male rats with 25 and 100 mg kg^{-1} DHEA. Arlt *et al.* (1999) observed a significant increase in circulating estradiol after oral administration of 50 or 100 mg DHEA to elderly men and Labrie *et al.* (2007) observed estradiol to be 158% over the basal level following exogenous DHEA application in postmenopausal women. Furthermore, the conversion of DHEA to other steroidal hormones depends upon the relative activity of key steroidogenic enzymes (Labrie *et al.*, 2007). Aromatase is a dual function enzyme which can catalyze the conversion of androstenedione to estrone and convert testosterone to estradiol (Payne and Hales, 2004). In this study, the 17β-HSD protein expression level increased while aromatase protein expression level decreased following treatment with DHEA at 24-48 h (Fig. 2b and c). The assumption can be made that androstenedione is preferentially converted to testosterone rather than to estrone due to up-regulation of 17β-HSD protein expression level and down-regulation of aromatase protein expression level. This was indirectly confirmed by the negative correlation that was evident between testosterone and estrone (Table 6). Bourguiba *et al.* (2003) demonstrated that androgens up-regulate the expression of aromatase in purified adult rat germ cells whereas estrogens have the opposite effect. Thus, based on the increased levels of estradiol, researchers believe that high estradiol concentrations result in down regulation of aromatase protein expression in TM-3 cells.

The progesterone content increased from 12-48 h following treatment with DHEA (Table 5) and correlation analysis revealed a positive correlation between progesterone and testosterone (Table 6). Pregnenolone is converted to progesterone and 17α-hydroxypregnenolone by 3β-HSD and CYP17α, respectively (Majewska, 1995). Progesterone was converted mainly to androstenedione and then translated into testosterone, its final metabolic product. Also, 17α-hydroxyprogesterone is translation into androstenedione or cortisol and then androstenedione converted to testosterone or estradiol. This could be explained by the fact that 17α-hydroxyprogesterone was not detected in the study. Arlt *et al.* (1999) reported that the serum concentration of cortisol was not altered by DHEA administration in healthy males for 3 days. Song *et al.* (2010) reported that the serum concentration of cortisone decreased at 24 h following intraperitoneal injection of DHEA in the male rat. The results showed that the concentration of cortisol, although minimal in all samples, decreased from 0-48 h and correlation analysis showed that cortisol was negatively correlated with testosterone and estradiol (Table 6). As noted above, 17α-hydroxyprogesterone can be converted to androstenedione or cortisol by CYP17α and CYP21A2, respectively. However, 17α-hydroxyprogesterone is prior to translation into androstenedione in the rodent and then converted to testosterone or estradiol (Brock and Waterman, 1999). Thus, the concentration of cortisol declined after DHEA incubation, likely due to a lack of precursor 17α-hydroxypregnenolone (Frye *et al.*, 2000), this was consistent with the correlation analysis between cortisol and both testosterone and estradiol. Also, researchers found that the CPY17α mRNA expression level increased in the treated group from 24-48 h compared to the respective control groups. It can speculated that decreased cortisol levels in TM-3 cells maybe due to the increased expression of CPY17α which catalyze 17α-hydroxypregnenolone translation into androstenedione and that this leads to a reduction in precursor availability for cortisol synthesis.

CONCLUSION

The present study demonstrated that DHEA is transformed predominantly into testosterone and estradiol, two end products in gonadal steroid hormone metabolism, through the regulated expression of steroidogenic and metabolic enzymes in TM-3 cells. However, the mechanism by which DHEA affects steroidogenic enzyme expression in Leydig cells remains unclear and additional study is needed.

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