Implication of Monoamines, Cortisol, Brain Derived Neurotrophic Factor and Opioid Receptors Genes in the Depressive-like Effect of Isotretinoin in Adult Male Rats: Comparison with Clonidine

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The present study describes the effect of isotretinoin (7.5 mg/kg body wt/ day, orally for 28 days) or clonidine (0.8 mg/kg body wt/day, i.p. for 7 days) on the concentrations of dopamine (DA), norepinephrine (NE), serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA) and brain derived neurotrophic factor (BDNF) and activity of monoamine oxidase (MAO) in the hippocampus and frontal cortex of adult male albino rats. The cortisol level was determined in serum. Gene expression of mu (MORs) and Kappa (KORs) opioid receptors genes and DNA damage were measured in the hippocampus-frontal cortex rat brains. The results show that administration of isotretinoin or clonidine caused an increase in DA, NE, 5-HIAA and BDNF concentrations, and a decrease in MAO enzyme activity in both the hippocampus and frontal cortex. In the hippocampus and frontal cortex, the 5-HT concentration increased significantly following clonidine treatment but not significantly changed after isotretinoin treatment. The level of serum cortisol increased following treatment with either isotretinoin or clonidine. Significant increases in MORs and KORs genes expression levels as well as in DNA damage of hippocampusfrontal cortex brain areas were also seen in isotretinoin or clonidine-treated rats compared with control rats. These results provide evidence that the depressive-like effects induced by isotretinoin or clonidine are exerted through alteration in monoamines, brain derived neurotrophic factor and cortisol levels as well as in opioid receptors gene expression.

Key words: isotretinoin, clonidine, depression, monoamines, cortisol, brain derived neurotrophic factor, opioid receptors, gene expression, DNA damage.

Retinoids, including vitamin A and its derivative are increasingly recognized to play an important role in adult brain function (Mey and McCaffery, 2004; Lane and Bailey, 2005). Retinoids are lipid soluble and easily cross the blood brain barrier into the central nervous system (Le Doze *et al.*, 2000). Retinoids receptors are concentrated in the limbic areas, including the hippocampus and the medial prefrontal cortex, the cingulate cortex and subregions of the thalamus and hypothalamus (Mey and McCaffery, 2004). These areas have been hypothesized to play a role in depression (Bremner, 2002). Retinoid-signalling regulates several behaviors including locomotor behaviors, learning and memory, sleep and mood (Lane and Bailey, 2005; Tafti and Ghyselinck, 2007; O'Reilly *et al.*, 2008). Retinoids also influence neurochemical systems that have been implicated in depression, in particular dopamine, serotonin and norepinephrine (Nestler and Carlezon, 2006; Arias-Carrion and Poppel, 2007; O'Reilly *et al.*, 2008).

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Isotretinoin (13 cis-retinoic acid) is a synthetic retinoid and is the active ingredient in Accutane, a widely prescribed oral acne medication (Zouboulis and Piquero-Martin, 2003). Administration of isotretinoin coincides with the onset of depressive symptoms in approximately 1-10% of patients (Hull and D' Arcy, 2005; Friedman et al., 2006; Kontaxakis et al., 2009), which were relieved following cessation of drug treatment. In animal studies chronic administration of isotretinoin has been shown to increase depression-related behaviors in both mice and rats (O'Reilly et al., 2006, 2008; Trent et al., 2009). However, the mechanism by which 13-cis-retinoic acid treatment can influence depression-related behavioral changes remains poorly understood.

Clonidine is an alpha₂-adrenoreceptor agonist used in the treatment of hypertension (Dollery, 1988). Central adverse effects such as sedation are the commonest problem. Depression is a known side effect of clonidine, the history of depression was short and it improved dramatically once the clonidine was withdrawn (Prasad and Shotliff, 1993). Clonidine has been shown to produce behavioral depressive effects in mice (Parale and Kulkarni, 1986) and rats (Rizk et al., 2009 a, b; Zaki and Rizk, 2013), and these animals have been suggested to be suitable animal models for depression. The biochemical basis for clonidineinduced behavioral changes in animals is thought to be due to a decrease in noradrenaline release in the central nervous system (Enginar and Eroglu, 1990).

The limbic-hypothalamic-pituitaryadrenal (HPA) axis circuit is involved in the central regulation of stress response. During stress response including depression, the hypothalamus secretes corticotropin-releasing hormone, which stimulates the anterior lobe of the pituitary gland to release adrenocorticotropic hormone, which stimulates the cortex of the adrenal gland to release cortisol (O'Connor et al., 2000).

Brain-derived neurotrophic factor (BDNF) is one of the most studied molecular substrates of hippocampal synaptic adaptations and neurogenesis. BDNF promotes proper spine density in the hippocampus, mediates antidepressant responses, and regulates depressive-like behaviors (Castren and Rantamaki, 2010).

The roles of opioid receptors in pain and addiction have been extensively studied (Bodnar, 2011), but their function in mood disorders has received less attention. Accumulating evidence from animal research reveals that mu, delta and kappa opioid receptors (MORs, DORs and KORs, respectively) are highly distinct players in the regulation of emotional states (Ribeiro et al., 2005; Knoll and Carlezon, 2010; Lutz and Kieffer, 2013). Opioid receptors are expressed throughout peripheral and central nervous systems (Le Merrer et al., 2009). The high density of opioid receptors in the limbic brain areas sets the opioid system as a central player in both reward processing and mood control, and a feasible target for treatment of emotional dysfunction (Berrocoso et al., 2009). Many neurotransmitter systems in the hippocampus and frontal cortex are involved in the pathogenesis of depression (Krishnan and Nestler, 2010) and opioid receptors regulate a number of these aspects (Lutz and Kieffer, 2013)..

The present study was performed to evaluate the depressant-like effect of isotretinoin in adult male rats and to compare this effect with that of clonidine, a suitable drug used as animal model of depression. Therefore, the following parameters that are thought to be implicated in depression were measured: monoamines level (DA, NE, 5-HT, 5-HIAA), MAO activity and BDNF concentration in hippocampus and frontal cortex and cortisol level in serum, as well as gene expression of mu and kappa opioid receptors genes and DNA damage in hippocampus-frontal cortex of adult rats. Besides food consumption and body weight were measured.

MATERIALSAND METHODS

Animals and Experimental Design

One hundred and five adult male albino rats of Wistar origin, weighing 125-145 g were used in this study. The animals were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR). They were housed under normal environmental conditions of temperature and humidity and allowed to adapt to the new environment for two weeks before starting the experiment. Animal rooms were maintained on a 12-h light, 12-h dark photoperiods. Animals were provided with food and water *ad. libitum*. All experiments were conducted in accordance with the NODCAR Guidelines for the Care and Use of Laboratory Animals.

Rats were randomly assigned into five groups, with twenty one rats in each group. Group 1:

Control group (Control). Rats received no treatment.

Group 2:

Control for clonidine-treated rats (Saline). Animals received a daily i.p. injection of normal saline (the solvent of clonidine) for 7 days. Group 3:

Clonidine-treated group (Clonidine). Rats received a daily i.p. injection of clonidine (Sigma Chemical Company) in a dose of 0.8 mg/kg body weight/day for 7 days. This dose was chosen according to study of Rizk et al. (2009 a, b). The authors indicated that i.p. injection of 0.8 mg/kg body weight for 7 successive days produced depression related behaviors in rats, represented by increase in total immobility time and decrease in struggling time in forced swimming test, a common animal model of depression.

Group 4

Control for isotretinoin-treated rats (Corn oil). Animals were orally administered 2ml/kg body weight/day corn oil (the solvent of isotretinoin) for 28 days.

Group 5

Isotretinoin-treated group (Isotretinoin). Rats received an oral oil solution of isotretinoin (Sigma Chemical Company) in a dose of 7.5 mg/kg body weight/day for 28 days. This dose produces serum levels of 13-cis retinoic acid similar to those of human Accutane® users (Ferguson et al., 2006). Food consumption pattern

Food consumption of rat per day was recorded daily during the period of treatment. Food consumption (g/rat/day) was calculated for each rat as:

Food consumption (g) = Food given (g) - Foodwasted (g)

Body weights

The body weights were recorded on the first day before treatment (initial) and on the day of sacrifice (final). The body weight was averaged for each week until the end of the treatment.

Blood samples and tissues preparation

In all experiments done the conditions

were adjusted to decapitate the animals between 3.00 and 4.00 pm. Twenty four hours following the last treatment, rats were sacrificed by decapitation and the trunk blood was collected and allowed to clot, then centrifuged. Serum samples were stored at -20°C for determination of cortisol level. Thereafter, the brain was rapidly excised and transferred to a dry ice-cold glass plate and the hippocampus and frontal cortex were rapidly excised, plotted dry on a filter paper to remove excess fluid and then weighed. Tissue samples were stored at -20 °C till taken for an analysis of monoamines content, monoamine oxidase activity and brain derived neurotrophic factor concentration. Brain samples (hippocampusfrontal cortex) used for RNA extraction were stored at -80°C, while that used for DNA extraction were stored at -20°C.

Biochemical analysis

Determination of serum level of cortisol

Cortisol level was determined in serum samples using enzyme linked immunosorbent assay (ELIZA) commercial kits (Immunospec Corp. USA).

Estimation of the amine contents

The estimation of DA. NE and 5-HT levels in hippocampus and frontal cortex of rats was carried out according to the fluorometric method described by Ciarlone (1978) and 5-HIAA level was determined by the method of Miller et al. (1970). Homogenization of tissues and recovery of the amines from tissue homogenates had been described previously (Chang, 1964; Ciarlone, 1974). Determination of monoamine oxidase activity

Monoamine oxidase (MAO) enzyme activity in the hippocampus and frontal cortex of rats was measured by the fluorometric method described by Olcese and De Vlaming (1979).

Determination of brain derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) concentration was estimated in the hippocampus and frontal cortex by enzyme linked immunosorbent assay (ELIZA) using rat specific commercial kits (Wkea Med Supplies Corp. CHINA).

Genetical analysis Gene Expression Analysis Isolation of Total RNA

TRIzol® Reagent (Invitrogen, Germany)

was used to extract total RNA from hippocampusfrontal cortex brain areas of rats according to the manufacturer's instructions with minor modifications. Briefly, tissue samples (50 mg) were homogenized in 1 ml of TRIzol® Reagent. Afterwards, the homogenized sample was incubated for 15 min at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then, the samples were vortexed vigorously for 15 seconds and incubated at room temperature for 3 min. The samples were centrifuged for no more than 12 000 g for 15 min at 4°C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a clean tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30°C for 10 min and centrifuged at not more than 12,000 x g for 10 min at 4°C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7500 g for 5 min at 4°C. The supernatant was removed and RNA pellet was air-dried for 10 min. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Isolated total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis (data not shown). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

Reverse Transcription (RT) Reaction

Complete Poly(A)⁺ RNA isolated from

brain tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Germany). An amount of total RNA (5 µg) was used with a master mix. The master mix was consisted of 50 mM MgCl₂, 10x RT buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 IU MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real Timepolymerase chain reaction (qRT-PCR).

Real Time-PCR (qPCR)

QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the brain cDNA copy number. PCR reactions were set up in 25 mL reaction mixtures containing 12.5 L 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 mL 0.2 mM sense primer, 0.5 mL 0.2 mM antisense primer, 6.5 mL distilled water, and 5 mL of cDNA template.

Two different amplification protocols were used for: 1) mu-opioid receptor (MORs), 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72 °C with 3 min for extension; and 2) kappa opioid receptor (KORs), 45 cycles of 1 min at 95°C, 1 min at 62°C, and 1.5 min at 72°C with 10 min for extension.

The quantitative values of RT-PCR (qRT-PCR) of MORs (MORs -F: 5' - AGT TCT GTA TCC CAA CCT CTT CC -3; , MORs-R: 5' -TCT GAC GAATTC GAG TGG AG -3' , Chavez-Valdez *et al.*, 2013) and KORs (KORs -F: 5' - GGA GAG AAT AGT AGC TGT ATG T-32 , KORs-R: 5' - AGC AGT ACC CTA AAA TGA TAT T-3' , Chavez-Valdez *et al.*, 2013) genes were normalized on the bases of β -actin (β -actin-F: 5' -TTG CCG ACA GGA TGC AGA A-32, β -actin-R: 5' -GCC GAT CCA CAC GGA GTA CT-3' , Girgis *et al.*, 2012) expression.

At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Calculation of gene expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet: Ef = $10^{-1/\text{slope}}$

Efficiency $(\%) = (Ef - 1) \times 100$

The relative quantification of the target to the reference was determined by using the $2^{\Delta\Delta CT}$ method if Ef for the target (MORs and KORs) and the reference primers (β -Actin) as follows:

 $\begin{array}{l} \Delta C_{_{T(test)}} = C_{_{T(target, \ test)}} \Delta C_{_{T(reference, \ test)}}, \\ \Delta CT(calibrator) = C_{_{T(target, \ calibrator)}} ``C_{_{T(reference, \ calibrator)}}, \\ \Delta \Delta CT = \Delta C_{_{T(Test)}} \Delta C_{_{T(calibrator)}}. \end{array}$

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

DNA damage of brain

DNA damage was measured using a single-cell gel electrophoresis (comet) assay.

The hippocampus-frontal cortex brain areas from rats were homogenized and isolated by centrifugation (15 min, 2000 r.p.m) in a density gradient of Gradisol L (Aqua Medica, Lodz, Poland). The concentration of the cells was adjusted to $(1-3) \times 10^5$ cells/ml by adding Roswell Park Memorial Institute medium (RPMI) 1640 without glutamine to the single cell suspension. A freshly prepared suspension of cells in 0.75% low melting point agarose (Sigma Chemicals) dissolved in phosphate buffer saline (PBS; sigma chemicals) was cast onto microscope slides precoated with 0.5% normal melting agarose. The cells were then lysed for 1h at 4°C in a buffer consisting of 2.5M NaCl, 100 mM EDTA, 1% Triton X-100, 10mM Tris, and pH 10. After the lysis, DNA was allowed to unwind for 40 min in electrophoretic solution consisting of 300mM NaOH, 1mM EDTA, pH>13. Electrophoresis was conducted at 4°C for 30 min at electric field strength 0.73 V/cm (30mA). The slides were then neutralized with 0.4M Tris, pH 7.5, stained with 2ug/ml ethidium bromide (Sigma Chemicals) and covered with cover slips. The slides were examined at 200 x magnification fluorescence microscope (Nikon Tokyo, Japan) to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV filter block consist an excitation filter (359nm) and barrier filter (461nm) and connected to a personal computer -based image analysis system, Lucia-Comet v.4.51. Fifty images were randomly selected from each sample and the comet tail DNA was measured (Blasiak et al., 2004). Endogenous DNA damage measured as the mean comet tail DNA of brain cells of the five rat groups (10 rats each). The number of cells scored for each animal was 100 (Blasiak *et al.*, 2004).

Statistical analysis

The values of the parameters determined are expressed as mean \pm S.E. Data were analyzed using the SPSS statistical package (version 18). Statistical significance was calculated using a oneway analysis of variance (ANOVA) followed by Duncan's Multiple Comparison post-hoc test. Values of p≤0.05 were considered statistically significant.

RESULTS

Biochemical analysis

Table (1) shows that, Food consumption was significantly decreased following treatment with both clonidine (-48.0%) and isotretinoin (-15.6%) when compared with the corresponding control. It is noticed that rats treated with clonidine consumed less food than rats treated with isotretinoin. The results also reveal that there were no differences in the initial body weight among the studied groups. Treatment with either clonidine for 7 days or isotretinoin for 4 weeks caused a significant reduction in the final body weights by -7.0% or -19.7%, respectively compared with their relative control groups.

The effects of clonidine or isotretinoin on the concentrations of DA, NE, 5-HT, 5-HIAA and activity of MAO as well as the concentration of BDNF in the hippocampus and frontal cortex of rats are represented in Tables 2 and 3. Serum level of cortisol is shown in Figure 1.

The concentration of DA, NE and 5-HIAA of clonidine-treated rats was significantly elevated in the hippocampus by 105.3%, 25.9% and 15.6%, respectively and in the frontal cortex by 26.3%, 30.8% and 21.1%, respectively compared to the relative controls. The same pattern of increase took place in isotretinoin-treated rats. This includes elevation in DA, NE and 5-HIAA by 40.6%, 29.1% and 14.0%, respectively in the hippocampus and 25.7%, 34.6% and 17.4%, respectively in the frontal cortex relative to their control groups. It's obvious from the results that in the hippocampus, the DA content in rats treated with clonidine was significantly higher than those who treated with

	Table 1. Effects o	of clonidine or isotreti	noin on food consumpti	ion and initial and	final body weight of a	dult male rats	
Groups Items	Control	Saline	Clonidine	% difference	Corn oil	Isotretinoin	% difference
Food	60.00 ± 0.00 ^c	60.00 ± 0.00 $^{\circ}$	31.2 ± 0.61^{a}	-48.0	$58.96\pm0.29~^{\circ}$	49.74 ± 0.87 ^b	-15.6
Initial body	231.9 ± 1.75	240.1 ± 1.64	241.1 ± 1.62	0.4	234.9 ± 3.93	230.8 ± 6.52	-1.7
weight (g) Final body weight (g)	$298.1 \pm 3.07^{\circ}$	258.7 ± 3.31 ^b	240.6 ±8.06 ª	-7.0	$314.5 \pm 5.0^{\text{ d}}$	252.6 ± 5.62 ^{ab}	-19.7
Data were expressed a a, b, c and d Means % difference: represe	as Mean ± S.E. The n s with different supers int a comparison betw Table 2. Effort	umber of animals was cripts, between groups, een control and treated fects of clonidine or i AAO) and brain deriv.	ten in each group. differ significantly (p≤0 d groups. souretinoin on monoam ed neurotrophic factor (.05). nines (DA, NE, 5-1 (BDNF) in the hip	HT and 5-HIAA), mon pocampus of adult ma	oamine le rats	
Groups	Control	Saline	Clonidine	%	Corn oil	Isotretinoin	%
Items				difference			difference
DA(µg/g)	$1.85\pm0.08^{\rm~a}$	$1.90\pm0.10^{\mathrm{a}}$	$3.90\pm0.16~^{\circ}$	105.3	$1.65\pm0.04~^{\rm a}$	2.32 ± 0.06 ^b	40.6
NE(µg/g)	0.54 ± 0.01^{a}	0.54 ± 0.01^{a}	0.68 ± 0.02 ^b	25.9	$0.55\pm0.01^{ m a}$	0.71 ± 0.03 ^b	29.1
5-HT(µg/g)	$0.52\pm0.02^{\mathrm{a}}$	$0.52\pm0.03^{\mathrm{a}}$	0.63 ± 0.02^{b}	21.2	$0.63\pm0.02^{\mathrm{b}}$	$0.57\pm0.02^{ m ab}$	- 9.5
5-HIAA(µg/g)	0.47 ± 0.02^{ab}	$0.45\pm0.02^{\mathrm{a}}$	$0.52\pm0.02^{ m bc}$	15.6	$0.50 \pm 0.01^{\rm ab}$	$0.57\pm0.02^{\circ}$	14.0
MAO(µM/g/hr)	8.56 ± 0.19^{b}	8.84 ± 0.16^{b}	7.31 ± 0.08 ^a	-17.3	$8.62 \pm 0.22^{\mathrm{b}}$	7.02 ± 0.38 ^a	-18.6
BDNF(ng/L)	$1.47\pm0.02^{\mathrm{a}}$	1.46 ± 0.03^{a}	$2.58\pm0.11^{\circ}$	76.7	$1.50\pm0.04^{\mathrm{a}}$	2.20 ± 0.09 ^b	46.7

Data were expressed as Mean \pm S.E. The number of animals was ten in each group. a, b, c and d Means with different superscripts, between groups, differ significantly (p≤0.05). % difference: represent a comparison between control and treated groups.

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	oxidase ((MAO) and brain deri	ved neurotrophic factor	r (BDNF) in the fro	ontal cortex of adult ma	ıle rats.	
Groups Items	Control	Saline	Clonidine	% difference	Corn oil	Isotretinoin	% difference
DA(µg/g)	1.13 ± 0.03^{a}	1.14 ± 0.05^{a}	$1.44 \pm 0.04^{\rm b}$	26.3	1.13 ± 0.05^{a}	1.42 ± 0.04 b	25.7
NE(µg/g) 5-HT(µg/g)	0.26 ± 0.01^{a} 0.33 ± 0.01^{a}	0.26 ± 0.01^{a} 0.32 ± 0.01^{a}	0.34 ± 0.01 ° 0.38 $\pm 0.01^{\rm b}$	30.8 18.9	0.26 ± 0.01^{a} 0.32 ± 0.01^{a}	0.33 ± 0.01^{a}	34.0 3.1
5-HIAA(µg/g)	$0.17\pm0.01^{ m a}$	0.19 ± 0.00 ^a	0.23 ± 0.01 ^b	21.1	$0.23 \pm 0.01^{ m b}$	$0.27\pm0.02^{ m c}$	17.4
MAO(µM/g/hr)	9.01 ± 0.09 ^b	8.99 ± 0.19 ^b	7.85 ± 0.38 ^a	-12.7	8.71 ± 0.24 ^b	7.34 ± 0.33 ^a	-15.7
BDNF(ng/L)	1.18 ± 0.03 ^a	1.30 ± 0.10^{a}	$2.21 \pm 0.10^{\text{ b}}$	70.0	1.17 ± 0.03 ^a	2.26 ± 0.11 ^b	93.2
Data were expressed a, b, c and d Mear	as Mean ± S.E. The as with different super	number of animals was rscripts, between group	s ten in each group. s, differ significantly (p ⁵	≤0.05).			

difference: represent a comparison between control and treated groups.

%

Table 3. Effects of clonidine or isotretinoin on monoamines (DA, NE, 5-HT and 5-HIAA), monoamine

isotretinoin. Serotonin content in the hippocampus and frontal cortex was increased significantly following clonidine treatment (21.2% and 18.9%, respectively), however no significant change was observed following isotretinoin treatment.

Administration of either clonidine or isotretinoin significantly decreased the activity of MAO in the hippocampus (-17.3% or -18.6%, respectively) and frontal cortex (-12.7% or -15.7%, respectively) compared to their corresponding control. Moreover, the concentration of BDNF was significantly elevated in both clonidine and isotretinoin-treated rats in the hippocampus (76.7 and 46.7%, respectively) and frontal cortex (70.0% and 93.2%, respectively) when compared to their relative controls. In the hippocampus the BDNF concentration in rats treated with clonidine was significantly higher than those rats treated with isotretinoin.

The results presented in Figure 1 show that serum cortisol level was significantly increased in clonidine (94.7%) and isotretinoin (175.6%) treated groups compared to the corresponding controls (Figure 1).

Expression analysis of MORs and KORs genes

Results of the expression analysis of MORs and KORs genes in brain tissues of rats are shown in Figures 2 and 3. The results showed that MORs and KORs genes in the brain tissues collected from rats treated with saline solution and corn oil were down-expressed $(1.12\pm0.02; 1.19\pm0.01,$ respectively) reaching similar level to control rats (1.09 ± 0.02) . However, rats treated with clonidine or isotretinoin showed significantly high expression levels of MORs and KORs genes compared with control rats (Figures 2 and 3). The expression levels of MORs and KORs genes in rats treated with clonidine were relatively higher $(1.73\pm0.04, 2.18\pm0.06)$ than those of rats treated with isotretinoin (1.66±0.05, 2.08±0.04, Figures 2 and 3).

The rate of DNA damage in hippocampusfrontal cortex of rats treated with saline solution or corn oil induced low rates of DNA damage which were relatively similar to that of control rats (Table 4). As the rates of DNA damage in rats treated with saline solution and corn oil were 4.6 and 5.0 %, respectively compared with 4.4 % in control rats. On the other hand, treatment of rats with clonidine or isotretinoine induced higher rate of DNA

Treatment	No. of cells		Class [¥] of comet]	DNA damaged
	Analyzed*	Total comets	0	1	2	3	cells (%)
Control	500	22	478	17	5	0	4.4
Saline	500	23	477	16	7	0	4.6
Clonidine	500	67	433	24	23	20	13.4
Corn oil	500	25	475	18	7	0	5
Isotretinoin	500	61	439	22	20	19	12.2

 Table 4. Visual score of DNA damage in hippocampus-frontal cortex

 of rats treated with clonidine or isotretinoin using comet assay

v: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No of cells analyzed were 100 per an animal. Number of animals: 5 animals per group.



Fig. 1. Effects of clonidine or isotrtinoin on serum cortisol level of adult male rats. Means with different letters, within groups, differ significantly ($P \le 0.05$)



Treatment

Fig. 2. Effects of clonidine or isotretinoin on the expression of mu (MORs) gene in hippocampus-frontal cortex of rats using quantitative Real Time-PCR analysis. Means with different letters, within groups, differ significantly ($P \le 0.05$)





Fig. 3. Effects of clonidine or isotretinoin on the expression of Kappa (KORs) gene in hippocampus-frontal cortex of rats using quantitative Real Time-PCR analysis. Means with different letters, within groups, differ significantly ($P \le 0.05$).



Fig. 4. Visual score of intact DNA (class 0) and DNA damage (class 1-3) in rat brain tissues treated with clonidine and isotretinoin using comet assay

damage than that found in control rats. The rates of DNA damage were 13.4% and 12.2% in rats treated with clonidine or isotretinoin, respectively (Table 4).

DISCUSSION

The present study indicates that daily administration of isotretinoin (7.5mg/kg body wt,

orally for 28 days) and clonidine (0.8mg/kg body wt, i.p. for 7 days) produced marked increases in the concentrations of DA, NE and 5-HIAA as well as BDNF and decrease in the activity of MAO enzyme in the frontal cortex and hippocampus of adult male rats.

Several reports have described isotretinoin effects on rodent central nervous system (Ferguson *et al.*, 2005 and O'Reillly *et al.*,

2006, 2008). Ferguson *et al.* (2005) reported that administration of 7.5mg/day 13-cis retinoic acid for 28 days resulted in a rise in DA and 5-HT metabolites in the striatum, a region that has been implicated in mood disorders. Further, systemic treatments with isotretinoin alters DA release in the prefrontal cortex (Jentsch *et al.*, 2000), increases intracellular 5-HIAA *in vitro* (O'Reilly *et al.*, 2007) and *in vivo* (Ferguson *et al.*, 2005), and elevates DA, NE and 5-HIAA in cerebral cortex, thalamushypothalamus, midbrain, cerebellum and ponsmedulla of adult male rats (Hassan *et al.*, 2008) as well as suppresses hippocampal neurogenesis (Crandall *et al.*, 2004).

In the present study, administration of isotretinoin did not cause significant change in 5-HT level in frontal cortex. Similarly, Ferguson *et al.* (2005) found no effects of 13-cis-retinoic acid on 5-HT level in hippocampus or frontal cortical brain homogenates. However, there was an apparent increase in 5-HT level in the striatum of adult male rats administered 13-cis-retinoic acid.

The current results also indicate that the alteration in monoamine concentrations caused by administration of isotretinoin or clonidine was accompanied by a decrease in food intake and body weight. These findings may be interpreted as signs of depression in rodents (Kopp et al., 1999; D' Aquila et al., 2000). These findings are similar to those obtained by several investigators (Booij et al., 2003 and Ruhe et al., 2007; Nutt, 2008) who noticed that alteration in DA, NE and 5-HT may causally be related to depressive disorders. Hassan et al. (2008) reported increased concentrations of DA, NE and 5-HIAA in discrete brain regions of adult rats following oral administration of isotretinoin for 4 weeks. The authors concluded that alteration in monoamine concentrations could contribute to the depressive-like effect induced by isotretinoin. Moreover, Rizk et al. (2009 a, b) and Zaki and Rizk (2013) demonstrated that administration of 0.8 mg/kg clonidine daily for 7 successive days produced depression-related behavior parallel to alterations in brain neurotransmitters in rats. Other studies indicated that depression induced by clonidine is probably due to a decrease in NE release in the central nervous system (Dennis et al., 1987).

From the present study, it can be suggested that the increase in DA and NE

concentrations in hippocampus and frontal cortex, following treatment with isotretinoin or clonidine, may reflect increased synthesis or decreased degradation of these amines. This was confirmed by the observed decrease in MAO enzyme activity, a mitochondrial enzyme responsible for the oxidative deamination of a variety of biogenic amines, following treatment with either isotretinoin or clonidine.

Zangen et al. (1999) reported that the increased tissue content of catecholamine (DA and NE) measured in some brain regions of a genetic rat model of depression, Flinders Sensitive Line (FSL) rats, might reflect either increased synthesis or decreased elimination of monoamines and metabolites via a decreased activity of MAO. However, a reduction in the elimination could be caused by a decreased release of amines and metabolites from nerve terminals. Also, a decreased amines release from nerve terminals may cause increased synthesis via a compensatory feedback mechanism (Galloway, 1990). Supporting the above hypothesis, Kvetnansky and Sabban (1993) reported that a variety of stress situations increases the activity of tyrosine hydroxylase, the first and major rate-limiting enzyme in catecholamine biosynthesis. Serova et al. (1998) reported increased tyrosine hydroxylase mRNA level in the ventral tegmental area of FSL rats as compared to control Sprague-Dawley rats.

In the current study, administration of isotretinoin or clonidine caused an increase in serum levels of cortisol. Several depressions are associated with hypersecretion of cortisol (Carroll *et al.*, 2012). The elevation of cortisol secretion in some depressive subtypes is thought to contribute to mitochondrial dysfunction, neuropathological changes, aging and other medical morbidity in individuals suffering from mood disorders (Gold *et al.*, 2002). Additionally, dysfunction in the neurotransmitters systems results, besides psychological and behavioral consequences, in the systemic effect with hyperactivation of stress hypothalamic-pituitary-adrenal axis (HPA) (Haddad *et al.*, 2002; Tafet and Bernardini 2003).

The present data demonstrate that treatment with isotretinoin or clonidine produced a significant increase in BDNF concentration in hippocampus and frontal cortex of rats. These results are not consistent with the findings of other researchers using models of stress-induced depression (Dell'Osso *et al.*, 2010; Shi *et al.*, 2010). Moreover, Crandall *et al.* (2004) and Sakai *et al.* (2004) reported that, retinoids are associated with an inhibition of neurogenesis, and cell proliferation in the hippocampus of mice.

However, Angelucci et al. (2000) reported higher BDNF levels in the frontal cortex, occipital cortex and hypothalamus of the 'depressed' FSL animals relative to the FRL controls. Tsai (2004) suggested that several processes could account for such discrepancies. For example, increased level of BDNF reflects a compensatory increase in its synthesis occurring in response to downregulation of the Trk-B signaling pathway in the genetic animal models of depression, or increased level of BDNF could reflect a lower turnover rate of BDNF with a higher pool of BDNF in the tissue that is not released. Castren et al. (2007) reported that mood disorders reflect failed function of critical neuronal networks, whereas a gradual network recovery through activitydependent neuronal plasticity induces the antidepressant-like effect.

BDNF and other neurotrophic factors have survival-promoting actions on a variety of central nervous system neurons, implying that a sustained reduction of these factors could affect neuronal viability. Increasing evidences suggest that neurotrophic factors inhibit cell death cascades by activating the mitogen activated protein kinase signaling cascade, and upregulating major cell survival protein such as B cell lymphoma-2, bcl-2 (Manji and Chen, 2002). The increased concentrations of neurotransmitters activate intracellular signal transduction cascades (Hashimoto et al., 2004), which result in enhanced expression of BDNF and its receptor (Duman et al., 2000; Hashimoto et al., 2004). Furthermore, there is evidence that these neurotrophic effects result in regeneration of catecholamine axon terminals in the cortex, enhance synaptic plasticity in the hippocampus, and may attenuate hippocampal atrophy (Duman et al., 2000). All monoamines (DA, NE, and 5-HT) are able to significantly increase BDNF astrocytes synthesis and secretion suggesting the existence for a positive reciprocal interaction between monoaminergic neuronal activity and astrocyte neurotrophic support in neuron-astrocyte

crosstalk which has a dynamic role in mediating neuronal plasticity and trophic functions in the brain (Juric *et al.*, 2006).

Based on the above reports, it may be suggested that elevation of BDNF in the hippocampus and frontal cortex of isotretinoin or clonidine-treated rats, found in the present study, may represent a compensatory response of neurons to overcome the decrease in neurogenesis processes.

The results of the current study revealed that rats treated with isotretinoin or clonidine showed significantly high expression levels of muopioid receptor (MORs) and kappa opioid receptor (KORs) genes in the hippocampus-frontal cortex brain areas compared with control rats. Researches on causal factors underlying mood disorders have mainly implicated MORs and KORs in the etiology of depressive states (Carr *et al.*, 2010; Lutz and Kieffer, 2013). This is thought to be, among other reasons, because MORs and KORs are densely distributed in several brain regions implicated in the response to stressors and emotionally salient stimuli (Berrocoso *et al.*, 2009).

The role of MORs in the pathophysiology of depression is controversial. In suicide victims who were mainly diagnosed with depression, MORs expression was increased in frontal and temporal cortex as well as in caudate nuclei (Escriba et al., 2004) compared with controls who died suddenly but with no history of psychiatric disorder. This suggests that depression and suicide are associated with higher MORs density. Moreover, some clinical reports describe the effectiveness of the MORs agonists, oxycodone and oxymorphine, in patients with refractory major depression (Bodkin et al., 1995; Stoll and Rueter, 1999). Filliol et al. (2000) and Yoo et al. (2004) reported decreased anxiety-and depressive-like behaviors in MORs knockout mice, indicating the possibility of a paradoxical depressant role of MORs in regulating emotional responses.

On the other hand, it has been shown that there is a pronounced reduction in MORs availability in the posterior thalamus and anterior cingulate cortex of patients with major depressive disorder that did not respond to treatment with the selective serotonin reuptake inhibitor fluoxetine (Kennedy *et al.*, 2006). Central administration of the endogenous peptides, endorphin-1 and endomorphin-2, which bind selectively to MORs receptors, decreased the immobility time in the tail suspension test and forced swimming test in mice without affecting motor activity (Fichna *et al.*, 2007). This shows that the activation of MORs underlies this antidepressant-like effect.

Acute pharmacological activation of the MORs reduces depressive-like behaviors in rodents (Berrocoso and Mico, 2009; Yang *et al.*, 2011; Berrocoso *et al.*, 2013). Whereas, chronic MORs signaling appears to be a major risk factor for depression (Hodgson *et al.*, 2009; Goeldner *et al.*, 2011).

Regarding the KORs, the current study showed that treatment of rats with isotretinoin or clonidine induced higher rate of KORs gene expression than that found in control rats. Activation of KORs produces depression or depressive-like behaviors in human and rodents (Nestler and Carlezon, 2006; Knoll and Carlezon, 2010; Deo et al., 2013), indicating that KORs agonists elicit depressant-like effects. Furthermore, KORs blockade or antagonists has antidepressantlike effects (McLaughlin et al., 2003; Shirayama et al., 2004; Zhang et al., 2007). Regarding knockout studies, it has been reported that disruption of the gene coding for the endogenous KORs agonist dynorphine, significantly reduced the time mice spent immobile in the repeated forced swimming stress test (McLaughlin et al., 2003, 2006).

Pearson *et al.* (2006) showed increased gene expression of the KORs in the locus coeruleus of Wistar Kyoto (WKY, rat strain is a putative genetic model of co-morbid depression and anxiety) rats compared to Sprague-Dawley rats. This result was confirmed by real-time PCR.

The KORs and the endogenous KORs ligand dynorphin are enriched in the ventral tegmental area, nucleus accumbens, and prefrontal cortex; brain regions that regulate mood and motivation (Berrocoso *et al.*, 2009). Neurochemical and electophysiological data have shown that KORs activation in these regions decreases DA transmission. KORs deletion or blockade, in the nucleus accumbens, increases basal DA release indicating the existence of a tonically active KORs system that inhibits basal mesoaccumbal neurotransmission (Shippenberg *et al.*, 2007). Decreased mesoaccumbal DA transmission is

implicated in certain symptoms of depression (Shippenberg *et al.*, 2007). Relevant to depression, opioid receptors also regulate the activity of the hypothalamus-pituitary-adrenal gland (HPA) axis, a major endocrine stress system (Knoll and Carlezon, 2010). Interestingly, opioid receptors regulate BDNF activity. Systemic administration of the KORs antagonist nor-binaltorphimine (Zhang *et al.*, 2007), had antidepressant-like effects in rats and increased BDNF mRNA in the hippocampus, as well as other brain structures (e.g., frontal cortex, amygdala, hippocampus, endopiriform cortex).

The present study revealed that treatment of rats with isotretinoin or clonidine induced higher rate of DNA damage in the hippocampus-frontal cortex than that found in control rats. In agreement with the present findings, Dygalo et al. (2004) reported that clonidine increased the level of apoptotic enzyme caspase-3 mRNA expression, and enhanced the DNA fragmentation in the brainstem of the 21-day-old fetuses and 8-day-old rats. Additionally, Georgala et al. (2005) reported that the marker of DNA damage in serum levels was two-fold higher in patients after isotretinoin treatment. Furthermore, a large body of evidence supports a role of oxidative stress in depression including increased peripheral markers of oxidative damage of lipids, proteins and DNA in depressed individuals, as well as reduced plasma antioxidants, antioxidant enzyme function and total antioxidant capacity (Maes et al., 2011). In clinical depression, increased serum levels of 8-oxo-7, 8-dihydro-2deoxyguanosine (8-oxodG), a marker of DNA damage from oxidation, have been reported (Forlenza and Miller, 2006). Moreover, a positive association between depressive symptoms and DNA levels of 8-oxodG in peripheral leucocytes in a non-clinical population (Irie et al., 2003), as well as in clinically depressed individuals (Irie et al., 2005) was reported.

CONCLUSION

This study provides evidence that isotretinoin induced a depressive-like effect, through its influence on some factors that have been hypothesized to play a role in depression in hippocampus and frontal cortex, brain regions that have been implicated in mood and emotional states. These factors include key neurotransmitters involved in depression, serum cortisol level, brain derived neurotrophic factor as well as mu and kappa opioid receptors gene expression and brain DNA damage.

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