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The effect of long-term exposure to combinations of growth promoters in Long Evans rats Part 2. Adrenal morphology (histopathology and immunochemical studies)

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Abstract

The aim of the study was to investigate the effects of long-term exposure (45 days) to growth promoters: clenbuterol (CB: 1 mg kg^{-1} bw) and/or dexamethasone (DEX: 0.1 mg kg^{-1} bw), in adrenal gland morphology, and the possibility of recovery after the withdrawal of drug treatment. Animals were sacrificed at different days of withdrawal (W0, W5, W10, W15 and W20), and adrenal glands processed for histopathology and immunohistochemistry. Adrenals of CB treatment showed typical features of long-term administration of β-agonists at W0 such as capillary dilatation in the fasciculata-reticularis zone, and this feature was also presented at W20. Adrenals of CB + DEX treatments showed the same results of CB treatment at days W0 and W20. However, DEX treatment presented the typical results of the exposure to corticoids with the atrophy of adrenal cortex. Immunohistochemistry of adrenal cortex steroidogenic enzymes (P450: scc, 3β-HSD, aromatase) denoted that neither positive staining nor localization was affected by treatments. Aromatase enzyme was immunolocalized in adrenal medulla cells in controls as well as in treated groups. The immunolocalization of glucocorticoid receptors showed an increase in CB (+++) and CB + DEX (++) treatments compared to the control group (0) and DEX treatment (0). Histopathological and immunohistochemical results are closely related to those found for adrenal endocrine function. We can conclude that chronic administration of growth promoters influence adrenal morphology and glucocorticoid receptor expression.

Keywords: Growth promoters; Clenbuterol; Dexamethasone; Adrenal histopathology; Immunohistochemistry; Steroidogenic enzymes; Rats

1. Introduction

The effects of long-term treatments of clenbuterol at several anabolic dosages have been studied on different organs and tissues of domestic and laboratory animal species [1–8]. However, there is still lack of information concerning the effects of combined growth promoters on the same organs and tissues [9,10] although consistent evidences on the use of these illicit cocktails in several species have been reported [11,12]. Our previous studies demonstrated that the administration of clenbuterol at growth promoting doses alone or combined with dexamethasone induced histological and endocrine change in ovarian function of rats leading to a disruption of reproductive function for a long time period [13,14]. We also stated adrenal cortex histological changes on rat adrenal gland after a 45-day treatment with clenbuterol (CB) [9]. Stereological studies of the adrenal gland of calves treated with clenbuterol presented an increase in the size of the nuclei of both adrenal steroidogenic cells and epinephrine-producing cells which is an index of adrenal gland hyperstimulation [15]. Our recently reported studies showed an endocrine hyperstimulation of the adrenal gland after the administration of clenbuterol alone or combined with dexamethasone (CB + DEX) both at cortical and medullary levels [16].

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Dexamethasone is a potent synthetic glucocorticoid and its effects on adrenal morphology/histopathology are contradictory, and/or it is dose-species dependent. Several authors have stated that it may produce irreversible morphological alterations on the adrenal gland through changes in the adrenal steroidogenic enzymes, adrenal steroidogenic receptors, adrenal vascularization, and by inhibiting hypothalamus–pituitary–adrenal (HPA) secretion/action [17–21]. In our above mentioned studies [13,14,16] it was found that while in the case of CB treatment endocrine hyperstimulation of both ovary and adrenal gland was reversed after a 20-day period of withdrawal, disruption was irreversible in both DEX and CB + DEX-treated rats.

The aims of the present study were to investigate whether the adrenal endocrine disruption (hyperstimulation) observed after the 45-day treatment of the β-agonist clenbuterol administered alone and combined with dexamethasone, is related to changes in adrenal morphology (adrenal vascularization), in the immunolocalization of adrenal steroidogenic enzymes, and in the immunolocalization/intensity of adrenal glucocorticoid receptors. A second step will be to investigate whether the morphological changes are reversible during a withdrawal period.

2. Experimental

2.1. Animals

The experimental protocols adhered to the Council of the EU rules [22] and were approved by the Institutional Animal Care and Use Committee of the Veterinary Faculty of Madrid at UCM (Spain). Treatments were previously described [13,14]; the study was performed on 100 right adrenal glands from those rats.

Briefly, rats were randomly allocated into 4 groups of 25 animals each. All animals were weighed weekly starting before the first administration and ending at sacrifice.

2.1.1. Control group

Twenty-five rats were dosed orally by stomach tube every day for 45 days with 1 mL of saline solution (SS).

2.1.2. Treatment I: clenbuterol (CB)

Twenty-five rats were daily dosed orally by stomach tube for 45 days with an anabolic dose of CB–SS (1 mL at a final concentration of 1 mg CB kg^{-1} body weight) (Clenbuterol hydrochloride, Sigma Co, St. Louis, MO, USA).

2.1.3. Treatment II: clenbuterol (CB) + dexamethasone (DEX)

Twenty-five rats were daily dosed orally by stomach tube for 45 days with the same dose of CB–SS administered in treatment I. Ten days before the end of treatment, rats were injected subcutaneously with 0.1 mg kg^{-1} of DEX (Decadrán, Merck Sharp & Dohme de España, SA).

2.1.4. Treatment III: dexamethasone (DEX)

Twenty-five rats were daily dosed orally by stomach tube for 45 days with 1 mL of SS. Ten days before the end of treatment rats were injected subcutaneously with 0.1 mg kg^{-1} of DEX.

Groups of 15 treated and 5 control rats were sacrificed at different days of withdrawal W0–W20: the day of sacrifice or 5, 10, 15, and 20 days after by means of cervical dislocation.

2.2. Histopathology and immunohistochemistry

Right adrenals were rinsed with 0.1 M PBS (phosphate buffered saline; pH 7.4) and placed in 4% paraformaldehyde fixative solution (pH 7.4), within 2 min of their collection, and left overnight. After fixation, the organs were placed in 0.05 M PBS for 12 h, and then dehydrated in a series of ethanol (30%, 50%, 70%, 95%, and 100%) embedded in paraffin wax and sectioned in 4 μm sections. All sections were stained with hematoxylin/eosin. All reagents were supplied by Sigma Co, St. Louis, MO, USA.

2.2.1. Immunohistochemistry of glucocorticoid receptor (GR)

Immunohistochemistry of GR was performed in samples ($n = 40$) of W0 and W20. Immunostaining was done on deparaffined 4 μm sections, placed in superfrost glass slides (Fisher Laboratories, CA, USA), using the streptavidin–biotin-peroxidase complex method, after a high temperature antigen unmasking protocol (boiling slides in a pressure cooker for 2 min in buffer citrate, pH 6). The slides were cooled down in distilled water and washed in Tris-buffered saline (TBS) (0.1 M Tris base, 0.9% NaCl, pH 7.4). Endogenous peroxidase activity was blocked in 1.5 mL H_2O_2/100 mL methanol for 15 min. GR immunostaining was performed by overnight incubation at 4 °C with polyclonal antibody (rabbit) Glucocorticoid receptor (1:40, Affinity Bioreagents, PA1-512). The slides were then incubated with anti-rabbit biotinylated secondary antibody (Vector Laboratories, 1:400, 30 min at room temperature). Afterwards, all the slides were incubated with streptavidin conjugated with peroxidase (Zymed, 1:400, 30 min, at room temperature). All washes and dilutions were made in TBS. The slides were developed for 10 min with a chromogen solution containing 3,3′-diaminobenzidine tetrachloride (Sigma Chemical Co.) and H_2O_2 in TBS. After washing in distilled water for 10 min, slides were counterstained in hematoxylin (Sigma), washed in tap water, dehydrated, cleared in xylene, and mounted. Negative control slides were made by substituting the primary antibody with TBS. Rat mammary gland tissue was used as positive control, according to manufacturer instructions. Adrenal gland was considered GR positive when more than 10% of positive cells were observed in 10 representative selected fields. Counting was done with a computer-assisted image analyzer (Olympus MicroimageTM image analysis, software Version 4.0 for Windows). Positive GR immunostaining intensity was also evaluated simultaneously by two observers, scored in each case as low (+), moderate (++), or intense (+++), based on the most frequent intensity found in the stained nuclei.