The effects of chronic and subtoxic chlorobenzenes on ACTH release

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Abstract: Many environmental chemicals and pesticides have been found to alter neuroendocrine communication in exposed biological objects. The environmental loads have primary and secondary effects that can alter the homeostatic regulation potential. Since it is difficult to avoid human exposition, a potentially important area of research to develop *in vivo* and *in vitro* experimental models. In this context, the primary aim of this study was to demonstrate effects of chlorobenzenes on adrenocorticotrophic hormone (ACTH) release. In our experimental study, male Wistar rats were exposed to 0.1, 1.0 and 10 µg / b.w.kg of 1,2,4-trichlorobenzene and hexachlorobenzene (ClB) mix via gastric tube for 30, 60 or 90 days. At the endpoints of the experiment blood samples were taken and animals were decapitated. Primary, monolayer adenohypophysis cell cultures were prepared by enzymatic and mechanical digestion. The ACTH hormone content in serum and supernatant media was measured by immuno-chemiluminescence assay. The Mg^{2+}-dependent ATPase activity was determined by modified method of Martin and Dotty. Significant differences were detected in the hormone release between the control and treated groups. The hormone release was enhanced characteristically in exposed groups depending upon the dose and duration of exposure. The Mg^{2+}-ATPase activity enhanced after chronic and subtoxic ClB exposition. Light microscopy revealed that the adenohypophysis seemed to be more abundant. Results indicate that Wistar rats exposed to subtoxic ClB have direct and indirect effects on hypothalamus-hypophysis-adrenal axis.

Keywords: chlorobenzenes, subtoxic**,** ACTH release, Mg^{2+}-dependent ATPase

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Introduction

Due to rapid industrialization acute and chronic poisoning with pesticides is a global public health problem. One group of pesticides is the persistent organic pollutants (POP), which can interfere with the endocrine communication that called endocrine disruptor compounds (EDC). These chemicals are xenobiotics that are proved to have effects on homeostasis and/or its regulation. The toxicity of POP depends on the effects of their hazardous nature, in combination with high chemical and biological stability, and a high degree of lipophilicity (Colborn et al., 1993). Various benzene derivates such as alkylbenzenes and chlorobenzenes (ClB), however, continue to be used as chemical intermediates, solvents, pesticides in spite of incomplete knowledge of their chronic toxicity. Several of the chlorinated benzenes are known to be porphyrogenic, carcinogenic, mutagenic and can interfere with the endocrine system in exposed animals and humans (Bigsby et al., 1999; Oehlmann et al., 2000).

Most papers showed only the contact and toxic effects of ClB, although these agents accumulate in adipose tissues and cause indirect alterations in exposed biological objects (De Bleckeer et al., 1992; Raymond-Delpech et al., 2005). ClB as stressors may activate the hypothalamic-pituitary-adrenal (HPA) axis in exposed mammals, which play role in the adaptation processes. Bousquet et al. (2001) showed that central and peripherally derived stressors activate neuroendocrine cascade mechanisms in the paraventricular nucleus. The corticotropin-releasing hormone (CRH) activates the corticotrophs cells in the anterior lobe of the pituitary gland, which stimulate the expression of proopiomelanocortin (POMC), the precursor of adrenocorticotropic hormone (ACTH) (Karalis et al., 2004; Liu et al., 2003). On the other hand there is grooving evidence that arginine-vasopressin (AVP) is involved in the physiologic regulation of ACTH via Ca^{2+} dependent receptor V1b (Gibbs et al., 1986; Legros, 2001; Lolait et al., 1995). The expressed ACTH is crucial for the development of adrenal cortex and play essential part in regulation of androgens and glucocorticoids (Beuschlein et al., 2001).

Reinhart (1988) showed that Mg^{2+} has both a direct effect on Ca^{2+}-channels and an indirect effect by stimulation of protein phosphatases that dephosphorylate Ca^{2+}-channels leading to enhance Ca^{2+} influx thus Mg^{2+}-dependent ATPase plays role in discrete energy transfer and in triggering exocytosis.

This paper focus on the central ACTH release in the pituitary by the effects of subtoxic exposition of ClB. We wanted to draw attention the effects of subtoxic exposition of ClB on

ACTH release and Mg^{2+}-dependent ATPase activity for this reason we aimed to develop a standardized *in vivo* and *in vitro* research model to study the basic regulation of ACTH. The aim of this study was also to investigate the structural and biochemical alterations of the anterior pituitary of male Wistar rats after subtoxic exposure to ClB.

1. Materials and methods

1.1 Animals

Male Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used in the experiment. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary. During the research period, rats were kept under controlled relative air humidity of 55-65% and 22±2℃, ambient temperature. Experimental animals lived under automated diurnal conditions (12 hr dark and 12 hr light system) in groups of 10 animals. Standard pellet food and tap water were available *ad libitum.*

1.2 Research protocol

Male Wistar rats were treated with combined ClB (1:1 mixture of 1,2,4- trichlorobenzene and hexachlorobenzene in 1 mL of 0.015% ethanol in distilled water was administered daily) in a dose of 0.1, 1.0 and 10.0 μg/b.w. kg via a gastric tube. The rats were exposed to ClB for 30 (n=10), 60 (n=10) and 90 (n=10) days. Control groups were set up: stress control (n=5, gastrostomy tube insertion group) and absolute control (n=5, untreated group). At the endpoints of the experiment (30, 60 and 90 days), blood samples were taken and serum was separated and stored at −70℃until measurements. After pentobarbital anaesthesia (4.5 mg/b.w. kg, Nembutal, Abbott, USA) the animals were killed and decapitated.

1.3 *In vitro* experimental model

Adenohypophysis and neurohypophysis were separated under a preparative microscope. Primary, monolayer adenohypophysis cell cultures (AdH) were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2% /Sigma, Hamburg, Germany/ for 30 min; collagenase /Sigma, Hamburg, Germany/: 30 μg/mL for 40 min; dispase /Sigma, Hamburg, Germany/: 50 μg/mL for 40 min in phosphate-buffered saline /PBS-A/; temperature: 37℃). Mechanical dispersion was achieved with nylon blutex sieves

(∅: 83 and 48 μm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2x10^5$/mL. The dissociated cells were placed onto 24 well-plastic plates (5% collagen coated /Nunc., Orlando, FL, USA/; Dulbecco's Modified Essential Medium /DMEM/ (Sigma, Hamburg, Germany) + 20% Fetal Calf Serum /FCS/ (Gibco, New York, NY, USA)+ antibiotics /Penicillin+Streptomycin: 1.0 μg/mL/). The cells were cultured at 37℃ in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking ACTH protein release. After functional standardization, the basal ACTH level was determined in Adh.

1.4 Adenohypophysis immunohistochemistry

The adenohypophysis was removed and separated from neurohypophysis, immediately dehydrated through an ascending ethanol series, fixed in 4% formalin solution and embedded in paraffin. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized 2 times with xylene (Sigma, Hamburg, Germany) for 10 min and 2 times with ethanol (Sigma, Hamburg, Germany) for 5 min. The deparaffinized sections were contour stained with hematoxylin and eosin. The indirect immunohistichemical method was carried out by incubating the sections overnight at room temperature using rabbit ACTH polyclonal antibody (dilution 1:500, Phoenix Pharmaceuticals, Inc.). Peroxidase activity was revealed with 0.04 % 4-chloro-1-naphtol (Sigma, Hamburg, Germany) and 0.001% hydrogen peroxide (Sigma, Hamburg, Germany) in Tris-HCl buffer.

1.5 Determination of ACTH and Mg^{2+}-ATPase activity measurement

The ACTH levels of blood serum and supernatant media were measured by LIA with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL) and DPC kit (L2KAC-02; Euro/DPC Ltd, Glyn Rhonwy, UK). The ACTH content was detected in the supernatant media. From the supernatant media, 500 μL samples were removed by Gilson pipette at appropriate times and stored at -80°C until immuno-chemiluminescence assay (LIA) was performed.

ACTH release of cultured corticotrophs is given in pg ACTH/mg protein. The ACTH content of serum is given in pg ACTH/ml serum.

A modified Lowry Method (Lowry et al., 1951) and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

The Mg^{2+}-dependent ATPase activity after ClB treatment was measured by the modified method of Martin and Dotty (Martin and Dotty, 1949). ATPase activity is expressed as control %.

1.6 Statistical analysis

To compare the means of different treatment doses (0.1, 1.0, 10.0 µg/b.w. kg) to the controls during 30, 60 and 90 days long treatments (n=10 in each group of time and dose) two-way ANOVA were run. Dose and time were used as the two factors for analyses.

For all three variables (ACTH levels of cell cultures, ACTH levels of serum and Mg^{2+}-ATPase activity) two-way ANOVA resulted a significant p-value ($p<0.001$), reflecting, there is an overall difference between the group means. As significant interactions were found between the two investigated factors (time and dose, $p<0.001$), these effects cannot be reported independently (so dose is changing with time, not separate effects).

Although owing to significant interaction, group differences could be examined separately with pairwise comparisons of group means based on estimated marginal means with Sidak adjustment for multiple comparisons. These pairwise comparisons were performed for all time groups (30, 60 and 90 days long) and revealed that all dose groups (0.1, 1.0, 10.0 µg/b.w. kg) differ significantly from control group for experimental groups ($p<0.001$).

Statistical analyses were carried out using IBM SPSS Statistics, version 21 (IBM Corporation, Armonk, NY, USA) software. All tests were two-sided, and $p<0.05$ was considered to be statistically significant.

2. Results

Our experiments revealed statistical differences between treated and untreated groups. In **Fig. 1** the serum ACTH content of absolute control groups were compared with that of the groups treated with 0.1, 1.0 and 10.0 µg/b.w. kg ClB. The ACTH levels of the groups treated with 0.1 µg/b.w. kg ClB mix were increased appreciably (96.55±0.37, 104.69±0.75, 117.47±0.87 pg ACTH/ml serum) compared with the control group (83.57±0.59 pg ACTH/ml serum). In the serum, the hormone content was increased (105.46±0.85, 120.46±0.73, 139.94±1.06 pg

ACTH/ml serum) significantly after treatment of 1.0 µg/b.w. kg ClB. Notable alterations were detected between the hormone secretion of control groups and the 10.0 µg/b.w. kg manipulated groups (117.91±1.14, 151.23±2.36, 167.44±1.38 pg ACTH/ml serum).

As shown in **Fig. 2**, the ACTH release of cultured adenohypohysis was increased significantly depending upon the dose and the duration of exposure. The secretion of ACTH was increased significantly as a consequence of 0.1 µg/b.w. kg ClB mix treatment (1621.7±1.01, 1628.8±0.93, 1669.2±1.05 pg ACTH/mg protein) compared with the control (1606.5±2.32 pg ACTH/mg protein). The hormone levels of supernatant media in 1.0 µg/b.w. kg ClB treated groups showed an elevating tendency (1675.2±1.60, 1698.3±1.80, 1669.2±1.67 pg ACTH/mg protein). As depicted in **Fig. 2**, the hormone levels in the 10.0 µg/b.w. kg ClB treated groups were elevated significantly (1715.3±1.48, 1738.4±1.15, 1756.8±1.19 pg ACTH/mg protein) compared with the control group.

As shown in **Fig. 3**, Mg^{2+}-ATPase activity was increased significantly (109.5±1.55, 114.9±1.59, 126.9±2.66%) by the effects of 0.1 µg/b.w. kg ClB mix treatment compared with the control (100%). Notable enhancement was observed in the enzyme activity (115.3±1.55, 121.9±2.63, 141.9±3.09%) after 1.0 µg/b.w. kg ClB treatment. A significant increase in ATPase activity (122.2±1.66, 136.6±1.66, 159.4±1.51%) was noticed by the exposition of 10 µg/b.w. kg ClB.

According to immunohistochemistry, the pituitary tissue in control groups revealed a normal histological structure. In contrast, in the ClB treated groups intense immunoreaction was observed for ACTH (**Fig. 4**).

3. Discussion

To investigate the effects of subtoxic ClB, *in vivo* model systems and *in vitro*, namely primary monolayer cell cultures were used and their cellular functions were standardized. We decided to focus on the alteration of the kinetics of ACTH release and the discrete energy transfer. The results arising from the current investigation reveal that the combined ClB treatment impairs the pituitary function.

The large number of pesticides is used worldwide although these compounds might threaten the environmental balance and stability of living organisms. In our experimental protocol ClB were used as model agents to present their endocrine disrupting effects. Our earlier studies showed that the applied dose of ClB mixture were subtoxic, because neither the body, the organ weight of Wistar rats and nor the aspartate aminotransferase, alanine

transaminase or gamma-glutamyl transferase results reveal any significant differences between experimental groups and control groups. No other malformations were observed (Valkusz et al., 2011).

In this context, HPA axis activation, which is under strong hypothalamic control, is crucial for stress response mechanisms. Under the control of hypothalamus and higher limbic brain structures the parvocellular neurons respond with CRH release into hypophyseal portal system, to act on POMC expressing corticotrophs and circulating ACTH. In addition AVP can potentiate the ACTH release via V1b receptors. We have postulated earlier that the subtoxic dose of ClB enhanced the AVP levels in the serum and in the supernatant media. The results showed increased ACTH levels in the serum and supernatant media. Our *in vitro* and histological staining experiments revealed structural alteration of the anterior pituitary suggesting the proliferative effects of subtoxic ClB. Indeed, we noticed pituitary β-cell hyperplasia, this observation may be caused by the enhanced levels of AVP and a response to CRH. Moreover, subtoxic exposure to ClB cause significant elevation in circulating ACTH in a dose and time dependent fashion. The released ACTH has crucial role in the regulation of adrenal cortex, which can manifest in number pathologies e.g. in adrenocortical adenomas (Sarkar et al., 2001; Bornstein and Chrousos, 1999) Laws et al. (2009) reported that the chlorotriazine herbicides, in toxic dose activate the ACTH dependent release of corticosterone in male Wistar rats.

Pollution monitoring method using enzyme activation and/or inactivation is proposed for studying polluted environments. It is known that cytoplasmic Mg^{2+} plays an important role in Ca^{2+} triggered exocytosis. Firstly, Mg^{2+}-dependent ATPase mediates the discreet energy transfer to potentiate exocytosis, secondly intracellular Mg^{2+} stabilize the vesicular protein complex (Wegenhorst et al., 1989; Torlinksa and Grochowalska, 2004). We found that ClB as xenobiotics altered the Mg^{2+}-ATPase activity due to interact directly with the enzyme. We found significant increase of Mg^{2+}-dependent activity during subtoxic ClB exposition, which can manifest in enhanced hormone exocytosis. Recently published literature has revealed that in corticotrophs the spontaneous electrical activity couples to hormone secretion (Stojilkovic et al., 2010). Calcium influx is involved in several signal transduction pathways that potentiate hormone release. Activated cell surface receptors mediate variable cellular processes, including G-protein utilization and cyclic mononucleotide accumulation. Activation of the Gq/G_{11} protein induces membrane-bound phospholipase-C that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield inositol-triphosphate (IP_3) and diacyl-glicerol (DAG) (Shipston and Armstrong, 1996; Rawling, 1996; Bauer et al., 2007). In

corticotrophs, IP_3 is essential to mediate the mobilization of non-mitochondrial Ca^{2+}. DAG activates Ca^{2+} dependent PKC and PKB, which phosphorylates Ca_V resulting in an increased Ca^{2+} influx: enhancing hormone exocytosis because of the activation of the SNARE complex (Brunger, 2000).

4. Conclusions

In conclusion, our *in vivo* and *in vitro* model is available to detect the effects of ClB on the hormone release of ACTH. Summarizing the above discussion, we found that Wistar rats exposed to subtoxic ClB have direct and indirect effects on HPA axis. Indirectly, the applied endocrine disruptor agents mediate the ACTH release. Directly, ClB enhance the activity of Mg^{2+}-dependent ATPase.

Declaration of interest

The authors report no declarations of interest.

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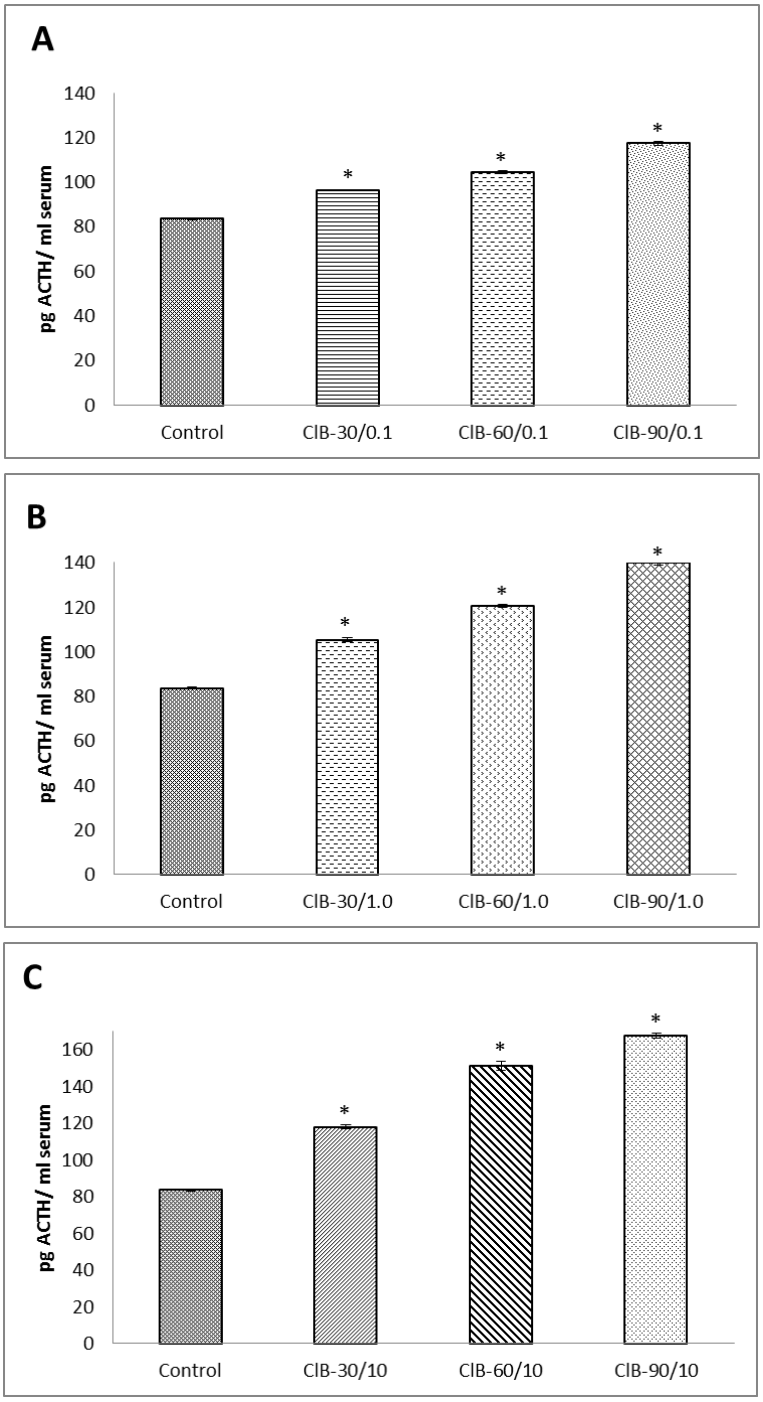

Fig. 1 ACTH content of serum after exposition of chlorobenzene mix. A: Experimental groups exposed to 0.1 µg/b.w. kg dose of ClB. B: Experimental groups exposed to 1.0 µg/b.w. kg dose of ClB. C: Experimental groups exposed to 10.0 µg/b.w. kg dose of ClB. The asterisks indicate the statistical differences to control ($P<0.001$).

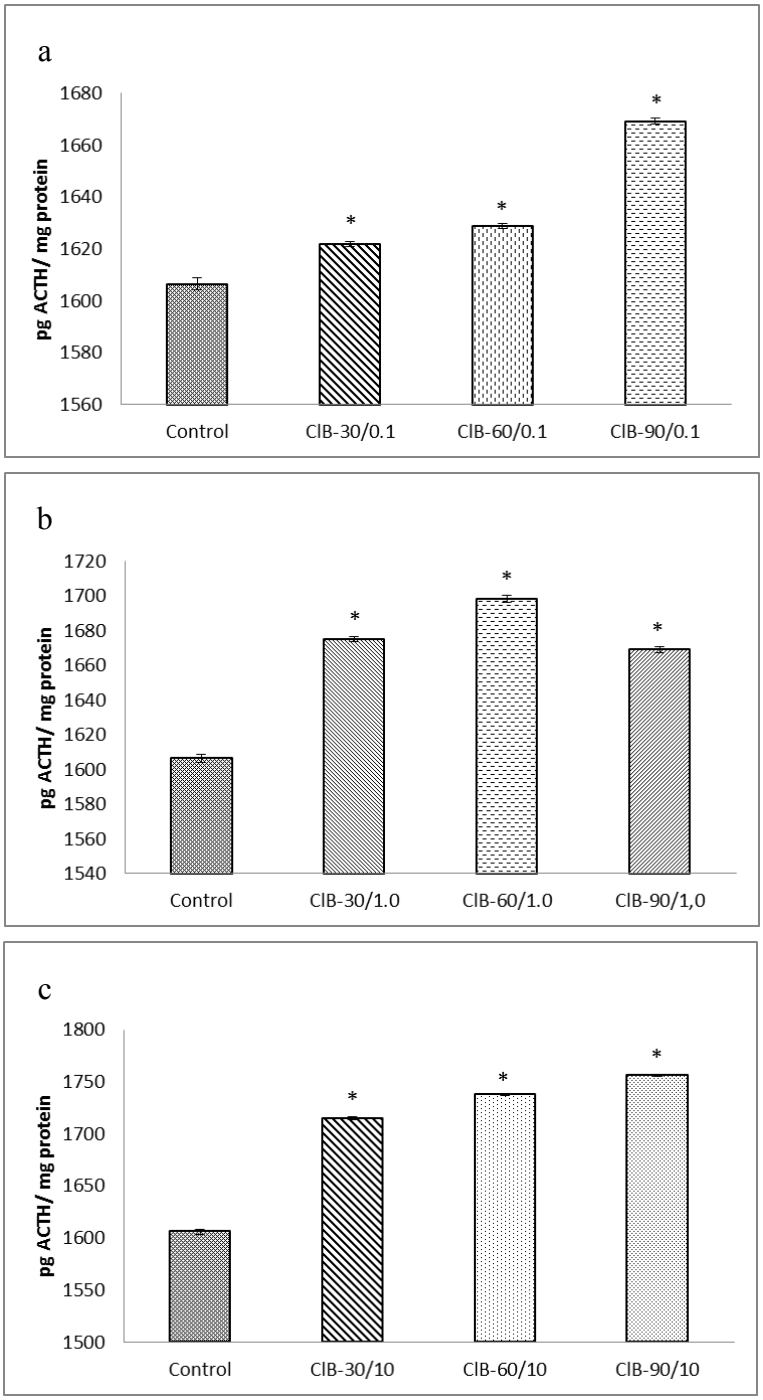

Fig. 2 ACTH expression of cultured adenohypophysis cell cultures after exposition of chlorobenzene mix. A: Experimental groups exposed to 0.1 µg/b.w. kg dose of ClB. B: Experimental groups exposed to 1.0 µg/b.w. kg dose of ClB. C: Experimental groups exposed to 10.0 µg/b.w. kg dose of ClB. The asterisks indicate the statistical differences to control ($P<0.001$).

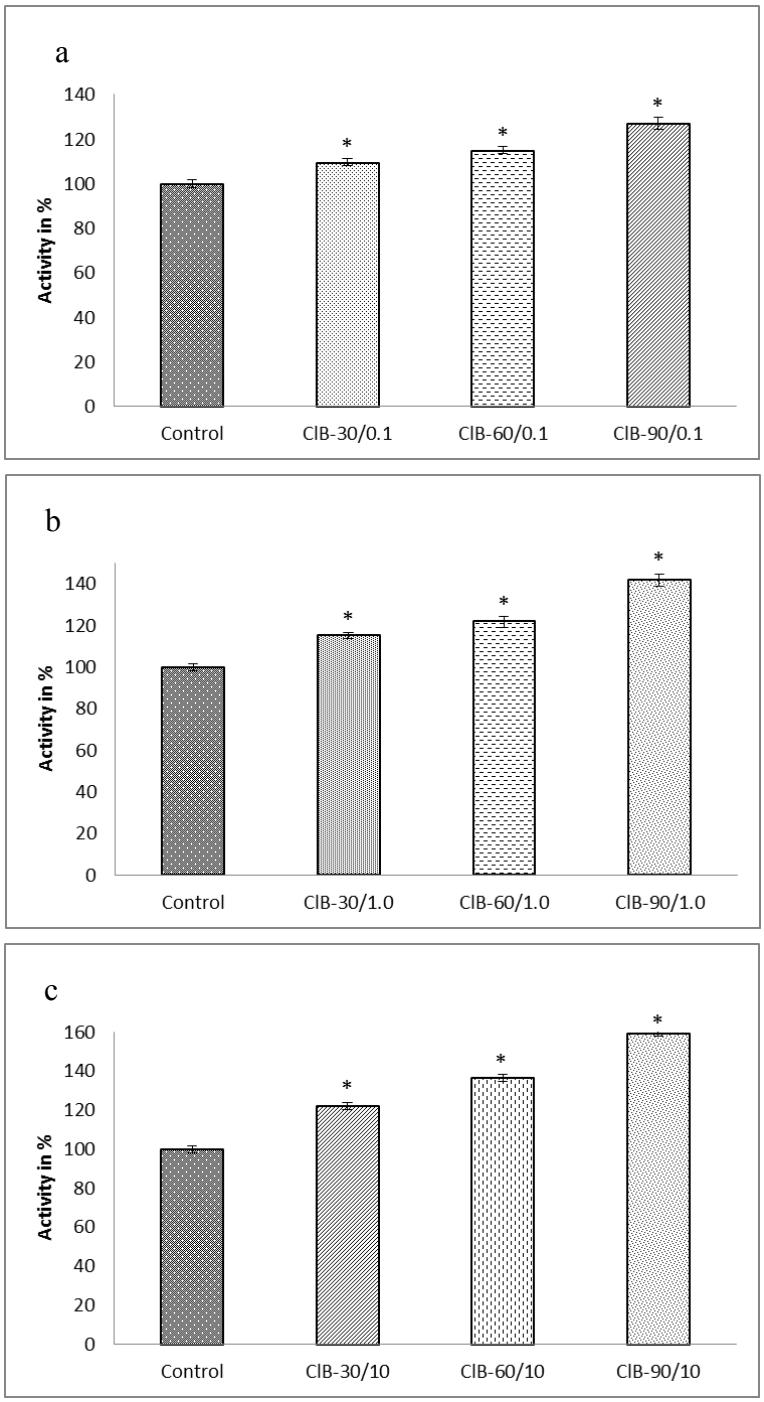

Fig. 3 The effects of subtoxic dose of chlorobenzene mix on Mg2+-dependent activity. A: Experimental groups exposed to 0.1 µg/b.w. kg dose of ClB. B: Experimental groups exposed to 1.0 µg/b.w. kg dose of ClB. C: Experimental groups exposed to 10.0 µg/b.w. kg dose of ClB. The asterisks indicate the statistical differences to control ($P<0.001$).

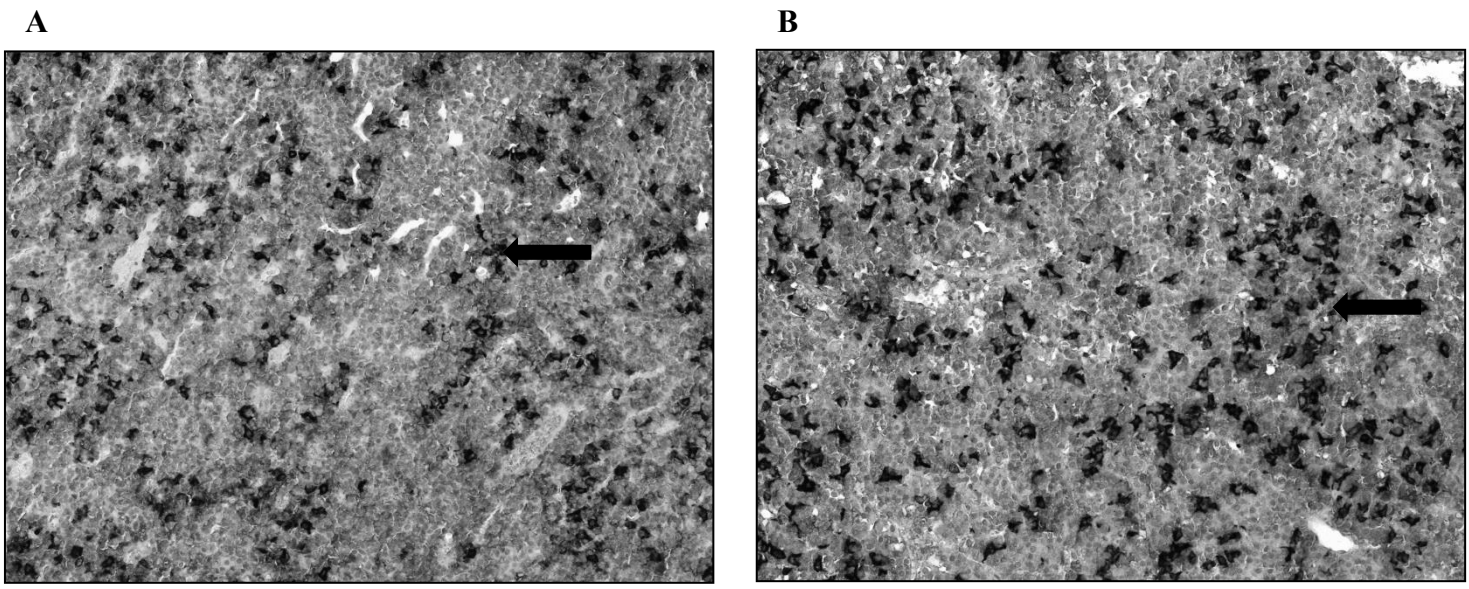

Fig. 4 Immunohistochemical analysis of ACTH expression in rat adenohypophysis. A: Presence of ACTH in control, untreated rat adenohypophysis tissue. B: Colocalization of ACTH in the 10.0 µg/b.w. kg dose of ClB treated rat adenohypophysis tissue (A-B: 20x). Arrows indicate ACTH secretion.