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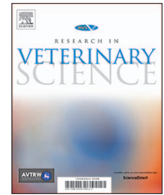

Influence of long-term, high-dose dexamethasone administration on proliferation and apoptosis in porcine hepatocytes

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ABSTRACT

The aim of this study was to examine the influence of long-term, high-dose dexamethasone administration on the liver, with particular emphasis on hepatocyte proliferation and apoptosis, using a swine model. The study included 48 large, female Polish breed pigs aged 3 months (weighing ca. 30 kg) divided into groups I (control; n = 24) and II (dexamethasone; n = 24) that receiving intra-muscular injections of monosodium phosphate dexamethasone for 29 days. The pigs were euthanized on days subsequent to the experiment. Immediately after the euthanasia, the pig livers were sampled, fixed, and processed routinely for histopathology, histochemistry, and immunohistochemistry (for proliferating cell nuclear antigen, Bcl-2, and caspase-3). Apoptosis was visualized by terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL). Dexamethasone administration gradually caused hepatocyte glycogen degeneration and finally lipid degeneration, accompanied by sinusoid and central vein dilatation and nuclear chromatin condensation. The proliferating cell nuclear antigen index, mean number of argyrophilic nucleolar organizer regions and proliferation index of argyrophilic nucleolar organizer regions were lower, while Bcl-2 expression was higher in group II compared with group I. The results from this study suggest that safe high-dose dexamethasone administration time is difficult to establish. Long-term, high-dose dexamethasone administration can cause pronounced morphological changes in hepatocytes by diminishing their transcriptional and proliferation activity but also protects them from apoptosis by potentially affecting Bcl-2 expression.

1. Introduction

Glucocorticosteroids (GCs) are steroid hormones produced by the adrenal glands that have a broad spectrum of activities, including regulating metabolism and immunomodulation (Ayroldi et al., 2012). Synthetic GCs are widely used as anti-inflammatory drugs and are invaluable for the treatment of many neoplastic and auto-immune diseases (Coutinho and Chapman, 2011). One of commonly used synthetic GCs is dexamethasone. The main action of dexamethasone is based on the down-regulation of genes that encode proinflammatory factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tumour necrosis factor alpha (TNF-α), or cyclooxygenase-2 (COX-2) (Barnes, 2011). Moreover, dexamethasone can also affect apoptosis in different ways. It was shown that it can either inhibit apoptosis in some cells, such as human neutrophils, tumour cells, and rat hepatocytes, or induce apoptosis in thymocytes, lymphocytes and multiple myeloma cells (Oh et al., 2006). In lymphocytes, dexamethasone has been shown to reduce the expression of nuclear and cytoplasmic microRNA processing enzymes, which increase GC-induced apoptosis (Gruver-Yates and Cidkowski, 2013). Furthermore, a previous report revealed that dexamethasone reduces the expression of pro-apoptotic Bax and increases the expression of anti-apoptotic Bcl-2 and Bcl-xl, leading to the inhibition of spontaneous apoptosis in human and rat hepatocyte cultures (Bailly-Maitre et al., 2001). In a rat hepatoma cell line, GCs can induce a reversible block in early G1 phase during the cell cycle. The synthetic GC dexamethasone binds to the cytoplasmic glucocorticoid-receptor-complex (GR) and translocates to the nucleus, where binds to cis-active response elements and results in cell cycle arrest or apoptosis (Sanchez et al., 1993).

While the immunomodulatory activities of dexamethasone are desirable in the context of their pharmacological application, overdosage or long-term administration can inhibit proper cell metabolism (Oray et al., 2016). Dexamethasone can influence some crucial functions of hepatocytes, including glucose metabolism, oxidative metabolism of xenobiotics by cytochrome P4503A (CYP3A4) induction and the urea cycle (Pascussi et al., 2001; Okun et al., 2015). GCs over-dosage can lead to hepatic enlargement, steatosis or glycogenosis, with massive glycogen storage in hepatocytes. The long-term administration of GCs

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Table 1
Primary antibodies used with the particular methods of antigen retrieval and visualization.

Primary antibody	Clone	Dilution	Incubation time	Source	Antigen retrieval	Visualization system
PCNA	Monoclonal mouse anti – PCNA, clone PC10	1:200	30 min in room temperature	DAKO, Denmark	2 × 3 min[c] TrisEDTA buffer pH = 9	EnVision + System-HRP, Mouse (DAB)[a]
Caspase-3	Rabbit polyclonal to Caspase 3	1:300	30 min in room temperature	Abcam, Great Britain	2 × 3 min[c] citrate buffer pH = 6	ImmPRESS Reagent Kit, Universal (DAB)[b]
Bcl-2	Rabbit polyclonal to Bcl-2	1:100	Overnight in 4 °C	Novus Biologicals, USA	2 × 3 min[c] citrate buffer pH = 6	ImmPRESS Reagent Kit, Universal (DAB)[b]

PCNA = proliferating cell nuclear antigen.
[a] Dako, Glostrup, Denmark.
[b] Vector, Burlingame, CA, USA.
[c] Antigen retrieval was conducted in a microwave oven, 650 W.

leads to an increase in glucose plasma levels, a decrease in its uptake in peripheral tissues and an increase in the supply of additional glucose to the liver for glycogen formation (Tamez-Pérez et al., 2015).

Dexamethasone also has cell proliferation and differentiation inhibitory effects. The inhibition of proliferation by dexamethasone is probably related to its anti-inflammatory properties. It has been shown that dexamethasone inhibited TNF-α and interleukin 6 (IL-6) expression after liver transplantation (Debonera et al., 2003). Interestingly, GCs decreased liver cirrhosis by inhibiting perivascular hepatic stellate cell activity and collagen synthesis in some autoimmune liver diseases, probably due to its anti-inflammatory effects (Czaja and Carpenter, 2004). Another study has shown that dexamethasone failed to suppress transforming growth factor beta 1 (TGFβ1) and increased alpha smooth muscle actin (α-SMA) expression in hepatic stellate cells, which consequently increased collagen α1 (IV), fibronectin and laminin production (Ki et al., 2005).

Long-term administration of synthetic GCs in high dosage, especially dexamethsone, is often used in neonatology to promote lung development, and prevent and treat acute and chronic lung injury in premature newborn infants during the neonatal period. In mature neonatal lungs changes form hyperoxia and barotrauma can be improved by treatment with dexamethasone (Holopainen et al., 2001; Carbone et al., 2012). In paediatrics high dexamethasone dosage can be used in different autoimmune and neoplastic diseases, mainly idiopathic thrombocytopenic purpura, acute asthma, and acute lymphoblastic leukemia (Shefrin and Goldman, 2009; Yadav et al., 2010; Kram et al., 2016). Moreover, in adult, treatment with synthetic GC is also very often prolonged, however in literature there is no evidence of dexamethasone safe administration time.

The proliferative activity of cells can be evaluated by numerous histochemical and immunohistochemical methods. The morphological criteria for proliferative activity include nuclear chromatin condensation, a number of argyrophilic nucleolar organizer regions (AgNORs) and a number of mitotic figures (Dörmer et al., 1984; Crocker and McGovern, 1988; Mikiewicz et al., 2016). There are several markers for cellular proliferation that can be assessed by immunohistochemistry, including proliferating cell nuclear antigen (PCNA). PCNA is crucial for proper DNA synthesis and repair and is expressed in late G1 and S phases in the cell cycle (Tanno and Taguchi, 1999; Webster et al., 2007). Previous reports have revealed higher PCNA expression in both hyperplastic and neoplastic processes in the liver (Tannapfel et al., 1999).

The balance between cellular proliferation and apoptosis determines proper organ functioning, including in the liver (Nagata, 1997; Bailly-Maitre et al., 2001, and Otrocka-Domagała, 2011). Cells undergoing apoptosis can be visualized using terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), which detects DNA fragmentation (Gavrieli et al., 1992; Slotta et al., 2009), or by immunohistochemical detection of executioner caspases (Eckle et al., 2004; De Minicis et al., 2012, and Zhang et al., 2013).

The aim of this study was to investigate the safe administration time and influence of the long-term, high-dose dexamethasone administration on the liver, with a particular emphasis on hepatocyte proliferation and apoptosis using swine model.

2. Materials and methods

2.1. Study design

This study was carried out in accordance with the principles for the care and use animals and was approved by the Local Ethics Committee for Animal Experiments (opinion No. 61/2010/N).

The study was conducted on 48 female, large Polish breed pigs aged 3 months and weighing ca. 30 kg. The animals were divided into the following two experimental groups: (Abdelhalim and Jarrar, 2011) group I (control) included 24 pigs and received no treatment and (Ayroldi et al., 2012) group II included 24 pigs and received monosodium phosphate dexamethasone (Rapidexon; NOVARTIS, Basel, Switzerland) administered intramuscularly for 29 days, with a daily dose of 1 ml/10 kg. The pigs were euthanized by intravenous injection of pentobarbital sodium salt (Morbital; BIOWET, Puławy, Poland) on days 16, 17, 18, 19, 20, 22, 25, and 29 (three pigs from each group/day).

2.2. Tissue sampling and staining

Immediately after euthanasia, the pig livers were sampled (1 sample each from the left lateral, left medial and right medial lobes), fixed in 10% buffered formalin, processed routinely for histopathology, embedded in paraffin and cut. The sections were stained with Mayer's haematoxylin and eosin (HE) and periodic acid-Schiff (PAS) according to McManus. Additionally, the nucleolar proteins associated with the nucleolar organizing regions (NORs) were stained via the silver method according to Ploton (Ploton et al., 1986) and the DNA distribution was visualized by Feulgen staining (Feulgen and Rossenbeck, 1924). For immunohistochemistry, the sections were mounted on silanized glass slides and subjected to heat-induced antigen retrieval (Table 1). The immunohistochemical examination was performed using primary antibodies (PCNA, Bcl-2, caspase-3) and a visualization system based on the immunoperoxidase method using 3,3-diaminobenzidine (DAB) as a substrate (Table 1). The slides were counterstained with Mayer's haematoxylin. Tissue sections were processed with the evaluated slides for the following markers as positive controls: (Abdelhalim and Jarrar, 2011) PCNA in the skin, (Ayroldi et al., 2012) Bcl-2 in B-cell lymphoma cells, and (Bailly-Maitre et al., 2001) caspase-3 in the tonsil. As negative controls, the primary antibody was either replaced with mouse IgG2a (DAKO, Glostrup, Denmark) at an appropriate dilution (for PCNA) or omitted (for Bcl-2 or caspase-3).

TUNEL staining for apoptosis was performed using the commercially available Trevigen Apoptotic Cell System (TACS) 2TdT-DAB in

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