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Effect of Fluocinolone-N treatment on fetal liver development in White Wistar Rats

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Abstract. Exposure to synthetic glucocorticoids during development can result in later cardiovascular and renal disease in sheep and rats. Although prenatal glucocorticoid exposure is associated with impaired renal development, less is known about effects on the developing liver. The main objective of this study is to analyze the side effects of glucocorticoid excess when treatment is done with Fluocinolone-N ointment, to see if it has any effect on rat newborns liver which have an important role in development of fetuses. Our results demonstrate that Fluocinolone-N treatment has negative impact upon the embryologic development of liver.

Keywords: glucocorticoid excess, fetal development, liver.

Introduction

Glucocorticoids are hormones that play a major role in energy homeostasis and stress response of the body (Vegiopoulus and Herzig 2007). As drugs they are most frequently used for immunosuppressive and anti-inflammatory purposes (Cosío *et al*., 2005; Newton *et al.,* 2010; Coutinho and Chapman, 2011; Spies *et al.,* 2011; Laugesen *et al.,* 2017). Glucocorticoids are used successfully in the treatment of a wide variety of diseases; however, some patients develop side effects (Crăciun *et al*., 1997a,b, 1998 a,b, 1999a,b,c, 2001; Rose *et al.,* 2010; Rebeyrol *et al.,* 2012; Woods and Weeks, 2005), while others fail to respond to this form of therapy (Schäcke *et al.,* 2002; Oppl *et al*., 2013). Fetal exposure to stress and its glucocorticoids hormone mediators exerts influences on organ growth, development and subsequent offspring physiology (Drake *et al*., 2007). In clinical situations, sources of maternal exposure to glucocorticoids includes; maternal stress, treatment with synthetic glucocorticoids in threatening preterm delivery and treatment of medical condition such as asthma (Singh *et al*., 2012).

Prenatal glucocorticoid exposure has been associated with a reduction in birth weight and postnatal alterations in glucose homeostasis and hypothalamic–pituitary–adrenal (HPA) axis function (Kapoor *et al.,* 2006; Kis *et al*., 1999, 2000; Sloboda *et al*., 2002; Jeje and Raji 2015; Paragliola *et al*., 2017). The mechanisms underlying these responses are unknown, although changes in fetal hepatic development may play an important role. The fetal liver produces key regulators of fuel metabolism and of the developing HPA axis, which are altered by hyperglucocorticoidemia (Fu *et al.*, 2016)

Animal studies have shown that prenatal exposure to synthetic GCs can have deleterious effects on the development of organs (such as the kidney, brain and the heart) which may in the longer term contribute to adult onset disease, including hypertension (Moritz *et al*., 2011; Gallo *et al*., 2012; Singh *et al*., 2012; Sullivan *et al.,* 2013). The role of synthetic glucocorticoids such as dexamethasone (DEX) in the programming of adult-onset diseases has been well studied (Jeje and Raji 2015). These studies have consistently demonstrated a reduction in nephron endowment following DEX treatment in sheep, spiny mice (Oppl *et al*., 2013). After glucocorticoid treatment similar changes can be observed in other mammals such as rats, rabbits and mice (Ortiz *et al*., 2003; O' Sullivan *et al*., 2013). Maternal administration of DEX for 48 h early in rat kidney development results in offsprings with a reduced nephron endowment. The authors hypothesized that DEX may indirectly inhibit nephrogenesis by inhibiting ureteric branching morphogenesis (Shingh *et al*., 2007). Damage to the kidney tissues may explain the hematological effect of DEX treatment. Chronic elevations in glucocorticoids induced by DEX are associated with increased liver fat (Harvey *et al*., 2017). Increased concentrations of glucocorticoids in humans and animals are associated with enhanced hepatic rates of lipogenesis and increased hepatic and plasma lipids (Amatruda *et al*., 1983; Beaudry *et al*., 2013; Drake *et al*., 2010; Yang *et al*., 2018). A number of facts studies suggest that some adult diseases may be the result of biochemical changes before birth produced by the elevation of glucocrticoid level (Romero and Butler 2007). Fetal organ systems are highly sensitive to changes in the intrauterine environment, including overexposure to glucocorticoids. Structural and functional alterations resulting from such changes may persist throughout life and have been associated with diverse diseases (Tegethoff *et al*., 2009). Antenatal corticosteroid therapy may increase the risk of cardiovascular disease in adulthood. Mice with global GR knockout have small and immature hearts that function poorly (Agnew *et al*., 2018).

Starting from the above observations, we can hypothesise that the abnormal hormone levels, can modify the program of the fetus development, causing such changes, which lead to dysfunction later in life. The mechanism of

glucocorticoid excess induced tissue damage has not yet been fully elucidated. In literature there are very few data about glucocorticoid excess and its relationship with embryological development. Therefore, the main objective of this study is to determine correlations in pregnant animals treated with Fluocinolon-N ointment, between glucocorticoid excess, viability of rat newborns and liver development.

Materials and methods

The experiments were carried out in pregnant (60-day-old) and newborn Wistar rats. The animals were kept under standardized bioclimatic conditions and fed on common rat chow, with water *ad libitum*.Commercial Fluocinolone-N ointment containing 25 mg Fluocinolone-acetonid-N/100 g excipient, was applied topically to the skin at 2 cm^2, for five consecutive days, by smearing 50 mg ointment/100 g b.w on the inguinal region. The daily dose of Fluocinolone-acetonid-N being equal to 12,5 g/100 g b.w. The newborn animals were divided into the following groups:

C-control group, newborns of untreated dams

FC1 – newborns of pregnant dams, treated with Fluocinolone-N in the 9^{th}- 13^{th} days of pregnancy

FC1- newborns of pregnant dams, treated with Fluocinolone-N in the 16^{th}- 20^{th} days of pregnancy

FC3 - newborns of pregnant dams, treated with Fluocinolone-N in the 9^{th}- 20^{th} days of pregnancy. These newborns did not survive 24 hours.

After 16 hours of fasting and 24 hours following the cessation of the treatment, the treated animals together with controls, were sacrificed by exsanguinations. The body and liver weights were measured with an accuracy of 0,00001 g immediately after excision. The significance levels were determined by parametric t-test. A $p<0,05$ was considered statistically significant. We measured parameters only from newborns which survived the first 24 hours after parturition, therefore only three groups of neonates were studied. We have no data from FC_3 newborns because none survived 24 hours after parturition.

For structural analysis the organs of the slaughtered animals were fixed in Bouin liquid and afterwards processed in view of being embedded in paraffin. The fragments were sectioned at the microtome with a thickness of 7 μ. The staining of liver was made by means of hematoxilin-eosin method (Muresan *et al.,* 1974)

Results and discussions

Viability and body weight of newborn rats

The parturition occurred on the twenty–first day of pregnancy. The autopsy was performed 24 hours after birth but not all infants survived the critical first 24 hours. At necropsy we found in mother's (FC2 and FC3) womb dead fetuses. Therefore we considered necessary to distinguish between fetuses, neonates and viable infants (Fig. 1).

The numbers of fetuses and newborns in the control and in the early stage of pregnancy treated FC1 group are equal, the viability of fetuses in these groups are approximately 100 %. The numbers of newborns in the FC2 and FC3 groups decreased considerable in comparison to control group. The viability of fetuses in the late stage of pregnancy treated group FC2 is approximately 66%, and in the FC3 is 0%.

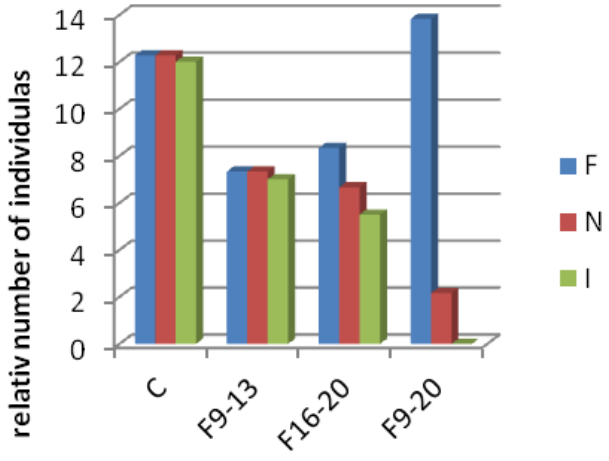

Figure 1. Relative number of foetuses (F), newborns(N) and viable infants (I)

The body weight of offspring FC1 was statistically equal to the control value (Fig. 2). Fluocinolone treatment did not cause long-term weight loss in newborns if the treatment was applied in the first stage og pregnancy. Nine days after treatment end the newborn's weight returned to the control value. In the late stages of pregnancy treated group (FC2) offspring body weight was significantly reduced compared with the control group. This means that glucocorticoid overdose of mothers in the late stage of pregnancy affected embryological development more than in FC1.

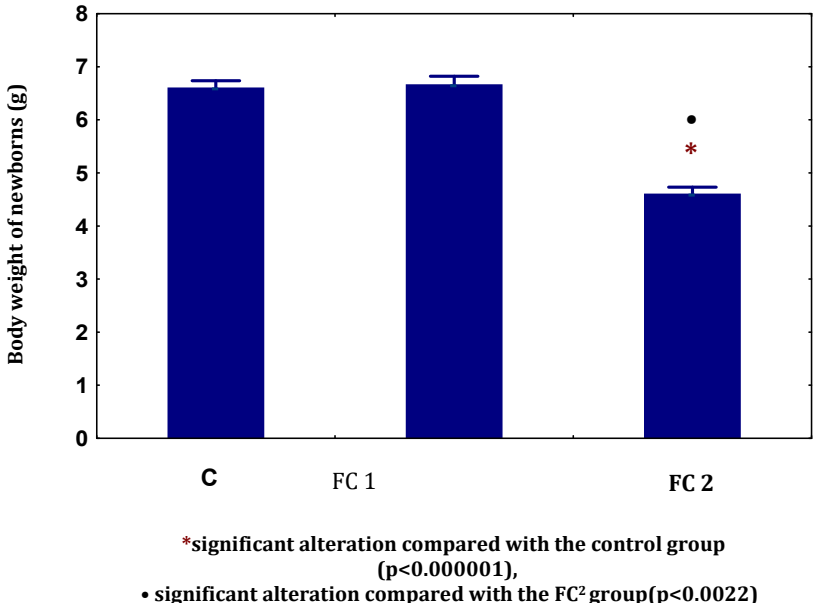

Figure 2. Newborns body weight after 24 h to parturition

The body weight of offspring FC1 statistically was equal to the control value. We could interpreted this observation in two ways: firstly the fluocinolone treatment did not cause long-term weight loss in newborns, if the treatment was applied in the first stage of pregnancy. Nine days after treatment end the newborn's weight returned to the control value. Secondly overdose of pregnant females with glucocorticoids did not affect the fetus development. In the late stages of pregnancy treated group FC2 offspring body weight was significantly reduced compared with the control group. This means that glucocorticoid overdose of mothers in the late stage of pregnancy affected embriological development more than in FC1. We could interpreted this observation also in two ways: firstly the glucocorticoid treatment caused weight loss but there was no time to returned to the control value because they were sacrificed, in the second way the glucocorticoid treatment in the late stage of pregnancy determined irreversible modification in fetus development. In these stage neurons develop more sensitive GR receptors, which cause stimulation of adrenergic neurons from brainstem, dopaminergic neurons from hypothalamus down regulated the secretory function of hipotalamo-hipophyseal axis.

The magnitude and nature of long-term effects of glucocorticoids might depend on tissue specific differences in ontogenetic expression of the GR (Bakker *et al*., 2001). Prenatal glucocorticoid treatment, is often GR specific, prenatal stress involves both MR and GR signalling and catecholamine release. And there is evidence that endogenous maternal and foetal glucocorticoids (and possibly other stress-related hormones) reduce birth weight and have implications for the developing foetal hypothalamic - pituitary – adrenal axis (Teghetoff *et al.,* 2009; Harris and Seckl, 2011).

The weight loss of newborns in conditions of glucocortioid excess were in consensus with our previous experimental findings that glucocorticoid treatment overregulated the somatotrope, tireotrope cells morphology and function (Kis and Crăciun, 2005).

Earlier studies with bethametasone have also observed reductions in birth weight of children exposed to prenatal corticosteroid treatment (Blomm *et al*., 2001, Pesonen *at al.*, 2009). The hypothalamic- pituitary-adrenocortical axis plays an important role in human behavior regulation and, thus, may have a role in explaining the associations of longer duration of betamethasone exposure with impulsivity and slower fetal growth with lower effortful control and higher negative affectivity (Seckl, 2008). There is evidence that endogenous maternal and fetal glucocorticoids reduce birth weight and have implications for the developing fetal hypothalamic-pituitary axis (Main *et al*., 2006; Harris and Steckl, 2011). The weight loss of newborns in conditions of glucocorticoid excess was in consensus with our previous experimental findings that glucocorticoid treatment overregulated the thyroid gland structure and function (Kis and András, 2017).

After fluocinolone treatment, the effect of glucocorticoids excess significantly reduced the liver weight in FC1 and FC2 groups compared with the control group (Fig. 3). In the early stages of embryonic development (FC1), the liver is less vulnerable to Fluocinolone-N ointment treatment, than in a later stages (FC2). FC^2 group presents a significantly alteration of liver weight compared with the liver weight in FC1 group.

In advanced stages of pregnancy, treated females offspring's are more sensitive to glucocorticoid excess. The placenta of dams in the later stage of pregnancy is more permeable to glucocorticoids. The excess of this hormone passes from the dam's body more easily and has a negative impact on the fetus liver development.

Histological study of the liver in controls and treated groups. Liver sections from control animals (Fig. 4a) displayed normal histological structure, the hepatic lobules have a visible connective tissue around and a relaxed structure. The lobules are roughly hexagonal, and consist of plates of hepatocytes radiating from a central vein. A distinctive component of a lobule is the portal triad (Fig. 4b), which can be found running along each corner of the lobule.

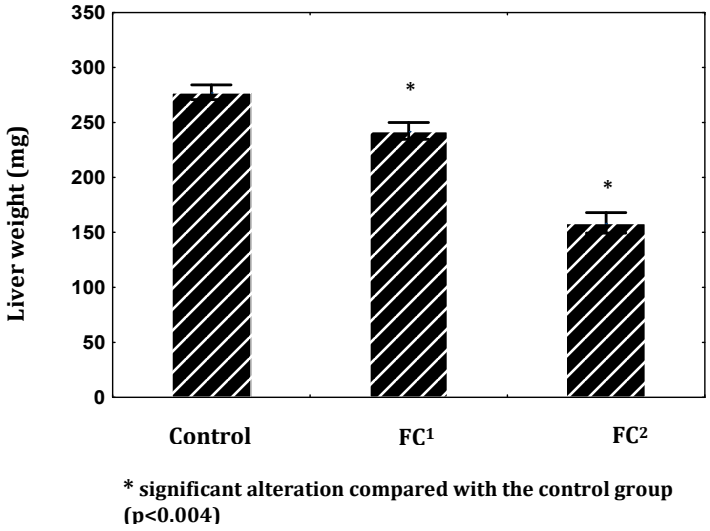

Figure 3. The newborn's liver weight

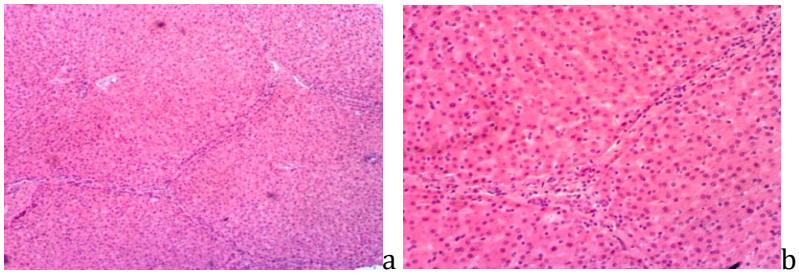

Figure 4. Control newborn's liver, a) Normal histological structure, 4x b) Relaxed structure of hepatic lobules, 20x

Between the hepatocyte plates are liver sinusoids, which are enlarged capillaries through which blood from the hepatic portal vein and hepatic artery enters via the portal triads, then drains to the central vein. In FC1 animals, liver tissue (Fig. 5a) is very similar to the liver tissue of control animals, the hepatic lobules can be well distinguished, but visible lipidosis can be observed in liver cells (Fig. 5b).

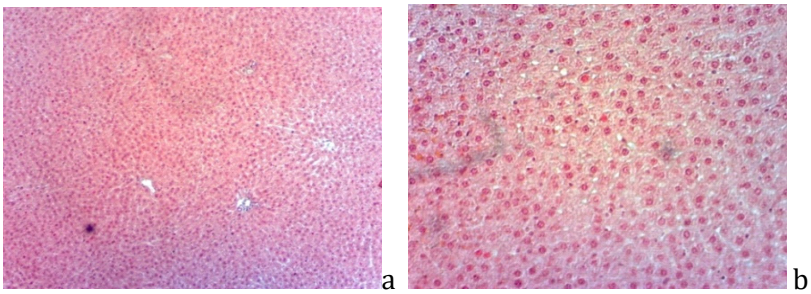

Figure 5. FC1 newborn's liver a) More condensate lobules 4x, b) Lipid droplets in the hepatic cells, 20x

Elevated glucocorticoids promote very-low-density lipoprotein production and secretion as well as triglyceride synthesis via fatty acid synthase and acetyl-CoA carboxylase. Along with the inhibition of free fatty acid oxidation via interference with the activity of Acyl-CoA dehydrogenase, glucocorticoids thereby, trigger hepatic fat accumulation (steatosis) as well as systemically elevated blood lipid levels (Crăciun *et al.,* 1998a, Vegiopoulos and Herzig, 2007, Woods *et al*., 2015). Clinical administration of exogenous glucocorticoids to patients, subjected to organ transplantation or suffering from severe inflammation-related diseases, is known to cause side effects, including deregulation of lipid metabolism and hepatic steatosis (Liu *et al.,* 2016). In the FC2 group, the liver sections of newborns are majorly different from the control animals (Fig. 6a), we can distinguish hepatic lobules, but they are more condensate than the control ones, the connective tissue underlying the lobules disappear definitively. We can see a hyperemia between the liver cells. In the zone of hyperemia the hepatic cells have granulo-vacuolar structure (Fig. 6b).

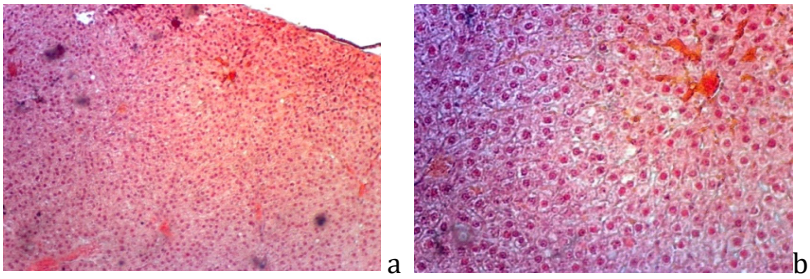

Figure 6. FC2 newborn's liver, 4x a) Condensate hepatic lobules with hyperaemia, b) Granulo-vacuolar structure of the hepatic cells, 20x

The presence of pyknotic cells in this liver zone indicates massive necrosis of liver cells, caused by the treatment with fluocinolone.

These histological changes of the liver tissues in the FC2 group are in consensus of alteration in liver weight. Literature data also show that excessive glucocorticoid level caused by glucocorticoid treatment affects the liver function. A significant proportion of patient with Cushing's syndrome will develop hepatic steatosis (Koliwad *et al*., 2009, Tarantino and Finelli, 2013). Glucocorticoid excess increases the circulating FFA and induce ectopic lipid accumulation in skeletal muscle and liver, all are associated with insulin resistance (Wang *et al.,* 2012).

Conclusions

The dose of Fluocinolone-N ointment used in the present study induced alteration of the rat fetus liver development.

The glucocorticoid excess induced by Fluocinolone-N treatment decrease the body and liver weight in fetuses of dam's treated in different stage of pregnancy.

The more affected animal group is the FC3, these did not survive 24 hours after the cessation.

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