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In utero exposure to dexamethasone programs the development of the pancreatic β- and α-cells during early postnatal life

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ABSTRACT

Aims: The aim of the present study was to clarify if *in utero* exposure to DEX would affect the development of different types of pancreatic endocrine cells during postnatal life.
Main methods: We investigated morphological and transcriptional features of both pancreatic β- and α-cell populations within the pancreatic islets during the early postnatal life of rats born to mothers treated with DEX (0.1 mg/kg) from day 14 to 19 of pregnancy. Untreated pregnant Wistar rats of the same age (12-week-old) were used as control (CTL). Pups were euthanized on the 1st, 3rd and 21st (PND1, PND3 and PND21, respectively) days of life, regardless of sex. Serum insulin and glucagon levels were also evaluated.
Key findings: Rats born to DEX-treated mothers exhibited increased pancreatic α-cell mass, circulating glucagon levels and *Gcg*, *Pax6*, *MafB* and *Nkx2.2* expression. Rats born to DEX-treated mothers also presented a rise in serum insulin levels on the PND3 that was paralleled by reduced β-cell mass. Such increase in serum insulin levels, instead, was associated with increased expression of genes associated to insulin secretion such as *Gck* and *Slc2a2*.
Significance: Altogether, the present data reveals yet unknown changes in endocrine pancreas during early postnatal life of rats exposed to DEX *in utero*. Such data may contribute to the understanding of the metabolic features of rats born to DEX-treated mothers.

1. Introduction

Observational studies show that intrauterine growth restriction (IUGR) increases the risk of type 2 diabetes (T2D) in adult life by impairing insulin sensitivity and insulin secretion [1–3]. Consistently, rats subjected to IUGR either by bilateral uterine artery ligation or by maternal protein restriction have been shown to develop glucose intolerance later in life [4,5]. Several studies in this field further support the notion that increased intrauterine exposure to glucocorticoids plays a pivotal role in the metabolic programming of IUGR [6–8].

Accordingly, offspring born to pregnant rats treated with dexamethasone (DEX) manifest IUGR, low birth weight and several metabolic alterations later in life such as glucose intolerance, increased hepatic glucose output and exacerbated hepatic lipid accumulation when exposed to fructose or prolonged fasting periods [9–13]. It was also described that treatment of pregnant rodents with DEX impairs the intrauterine development of endocrine pancreas leading to a reduction in the number and mass of pancreatic β-cell between the 15th and 21st gestational days [14,15]. Reduced insulin secretion and β-cell fractional area remain evident until 120 days of age in rats born to DEX-treated mothers [16].

Although the endocrine pancreas continues to develop during approximately 1 month after birth [17], few studies have investigated if antenatal exposure to DEX affects endocrine pancreas development during early postnatal life. In foals, *in utero* exposure to DEX did not significantly affect basal and glucose-stimulated insulin secretion [18]. On the other hand, it has been shown that pancreatic islets of rats exposed to DEX during fetal life have reduced number of PDX-1 positive cells and increased markers of oxidative stress on the 7th postnatal day [16]. Similarly, previous observations also showed that rats born to

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DEX-treated mothers have reduced number of pancreatic islets per pancreas section, islet size and pancreatic islet insulin content on the 7th postnatal day [19], and reduced pancreatic islet insulin expression on the 21st postnatal day [20].

It is noteworthy, however, that rats born to DEX-treated mothers do not display reduced *in vivo* insulin levels in spite of having glucose intolerance and increased hepatic expression of enzymes related to gluconeogenesis [9]. Thus, it is reasonable to hypothesize that in addition to pancreatic β-cells, *in utero* exposure to DEX may also modulate the postnatal development of pancreatic α-cell. Indeed, glucocorticoids receptor (GR) is equally expressed in both β- and non-β-cells of pancreatic islets during the postnatal life of mice [21].

Attempting to clarify if *in utero* exposure to DEX would affect different types of pancreatic endocrine cells during postnatal life, we have presently assessed morphological parameters of both β- and α-cell populations of rats born to DEX-treated mothers at multiple time-points before weaning. In parallel, we analyzed the expression of several genes related to β- and α-cell differentiation and secretory function.

2. Materials and methods

2.1. Animals

All experimental procedures followed the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the State University of Campinas Committee for Ethics in Animal Experimentation (protocol No. 4385–1). Female Wistar rats were obtained with 4 weeks of age from the Animal Breeding Center at the University of Campinas (CEMIB, Campinas, Sao Paulo, Brazil) and kept under a 12-h light–dark cycle at 22 ± 2 °C with standard chow and water *ad libitum*.

2.2. Glucocorticoid exposure

At 12 weeks of age, females were housed in individual cages with one male for 3 days. The presence of spermatozoa in a vaginal lavage indicated day 0 of gestation, the moment when pregnant rats were isolated. On the 14th day of pregnancy, half of the pregnant rats were treated with dexamethasone (DEX) (0.1 mg/kg body mass; Achê Pharmaceutical Laboratories, Guarulhos, SP, Brazil) diluted in the drinking water until the 19th day of pregnancy as previously described [22]. Briefly, daily liquid intake and body weight were daily monitored between the 14th and the 19th days of pregnancy. The liquid volume consumed by each rat was used to adjust the concentration of DEX that was necessary to provide the final dosage of 0.1 mg/kg/day.

Untreated pregnant rats were used as controls (CTL). On the next day after delivery, the number of pups was adjusted to six per lactating mother, and the remaining non-fasted neonates were used to obtain the data on the 1st (PND1) day of life. Non-fasted lactating pups were used for experimental procedures on the 3rd (PND3) and 21st (PND21) days after birth, regardless of sex. Lactating pups were weighted and euthanized by decapitation (previous sodium thiopental anesthesia [80 mg/kg] was used in pups at PND21) at the day of the experiments. Trunk blood samples were collected and the serum was separated by centrifugation. Thereafter, serum insulin (Merck Millipore, Billerica, MA, USA; cat. no. EZRMI-13K) and glucagon (R&D Systems, Inc., Minneapolis, MN, USA. cat. no. DGCG0) levels were quantified in duplicate using commercial ELISA kit, following the manufacturer's recommendations. Intra-assay and inter-assay variations (% CV) were respectively 1.33 and 6.71 for insulin (at the concentration of 2.32 ng/ml) and 3.3 and 8.7 for glucagon (at the concentration of 315 pg/ml). Glucose levels were assessed in whole blood using a glucometer (Accu-Chek Active, Roche, Basel, Switzerland).

Serum samples from pups belonging to the same litter were processed as a pool and considered as individual sample. For morphometric analysis of the endocrine pancreas, only one pup per litter was used independently in each specific postnatal time point.

2.3. Pancreas morphometry and immunohistochemistry

For morphometric analysis, pancreata of both groups of rats were removed, weighted, fixed for 24 h by immersion in 4% (wt/vol) paraformaldehyde fixative solution and routinely embedded in paraffin. From each block, exhaustive 5-μm serial sections were obtained (every 20th section) and randomly selected for insulin or glucagon immunoperoxidase reaction as previously described [22,23]. Briefly, the sections were incubated with a polyclonal guinea pig anti-insulin (1:400; Dako North America, Inc., CA, USA; cat. no. A0564) or rabbit anti-glucagon (1:200; Dako North America, Inc., CA, USA; cat. no. A0565) antibody at 4 °C overnight. After this period, sections were incubated either with HRP-conjugated anti-guinea-pig IgG (1:1000; Invitrogen, Carlsbad, CA, USA; cat. no. 614620) or biotinylated mouse anti-rabbit IgG, avidin and biotinylated HRP (Santa Cruz Biotechnology; St. Cruz, CA, USA; cat. no. SC-2018) for 2 h at room temperature. The positive insulin or glucagon cells were detected with 3,3′-diaminobenzidine solution (Sigma-Aldrich Co; St Louis, MO, USA). Finally, the sections were quickly stained with Ehrlich's hematoxylin and mounted for microscopy observation. All islets present in the sections were covered systematically by capturing images with a digital camera (Olympus DP72) coupled to a light microscope (Olympus BX51TF) under a final magnification of 20×. The islet, β-cell and α-cell section areas were obtained with ImageJ software (http://imagej.nih.gov/ij). The percentage of β- or α-cells were estimated by the ratio of the immunostained area to insulin or glucagon to the total area of the respective pancreatic islet × 100. The β- and α-cell masses were calculated by multiplying the pancreas weight by the total β- or α-cell area per pancreas section. All morphometric analysis of the endocrine pancreas were evaluated in 2 independent sections of each animal.

2.4. Islet isolation

Pancreatic islets were isolated by digesting the pancreata with Type V colagenase (Sigma-Adrich, San Luis, MO, USA) as described before [24]. To minimize the contamination with exocrine tissue, islets were cultured for 8 h in RPMI-1640 medium containing 5.6 mmol/L glucose (Vitrocell; Campinas, Sao Paulo, Brazil) supplemented with 5% fetal bovine serum (Vitrocell; Campinas, Sao Paulo, Brazil), at 37 °C, in a 5% CO_2 – air atmosphere. The islets were than individually collected under a light microscope, and used for mRNA expression analysis.

2.5. RNA extraction and qPCR

Total RNA was extracted from a pool of approximately 500 freshly isolated islets using a RNeasy Plus Mini kit (Qiagen, Hilden, Germany) as previously described [22]. Briefly, aliquots containing 2 μg of RNA were subjected to reverse transcription using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). PCR reactions were conducted using KAPA SYBRβ FAST qPCR Master Mix (Kapa Biosystems, Inc., Boston, MA, USA) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The specificity of the reactions was verified with melting curve analysis. The primer sequences used are as follows: *Ins2* sense: 5′-ACAAGTGGCACA ACTGGAGCTG-3′; *Ins2* antisense: 5′-GAGAGAGCAGATGCTGGTG CAG-3′; *Slc2a2* sense: 5′-TCTTCACGGCTGTCTCTG-3′; *Slc2a2* antisense: 5′-GCACAGAAAAACATGCCA-3′; *Gck* sense: 5′-GCACAAGTCGTACCA GCT-3′; *Gck* antisense: 5′-AAAGCTCAGCGAACCCCG-3′; *Ngn3* sense: 5′-GTCGGGAGAACTAGGATG-3′; *Ngn3* antisense: 5′-AAAAGGTTGTTG TGTCTCTG-3′; *Neurod1* sense: 5′-TTTCTTGCCTGAGCAGAATCCG-3′; *Neurod1* antisense: 5′-ACCCGAGGAGAAGATTGATCCG-3′; *Pdx1* sense: 5′-AACGTAGTAGCGGGACAACGAGC-3′; *Pdx1* antisense: 5′-GAGGTGG TGGCTTTGGCAATG-3′; *Pax4* sense: 5′-CTGTGGTGGCTCGAATTGCC-3′; *Pax4* antisense: 5′-GGAGAAGATAGTCCGATTCC-3′; *Gcg* sense: 5′- CGC

CAGATCATTCCCAGCTTCC-3′; *Gcg1* antisense: 5′-CGCCCAAGTTCCTC AGCTATGG-3′; *Pax6* sense: 5′-ATGCCCAGCTTCACCATG-3′; *Pax6* antisense: 5′-GAACTGACACTCCAGGTG-3′; *Brn4* sense: 5′-GGCTGATTCAT CCACAGGAAG-3′; *Brn4* antisense: 5′-TTCCAGTACGCCCTTGACACT-3′; *MafB* sense: 5′-GACGCAGCTCATTCAGCAG-3′; *MafB* antisense: 5′-CCG GAGTTGGCGAGTTTCT-3′; *Nkx2.2*sense 5′-CAGCAGCGACAACCCC TAC-3′; *Nkx2.2* antisense: 5′-AAGAGCACTCGGCGCTTCC-3′; *Nkx6.1* sense: 5′-ATGGGAAGAGAAAACACACCAGAC-3′; *Nkx6.1* antisense: 5′-TAATCGTCGTCGTCCTCCTCGTTC-3′; *Foxa2* sense: 5′-CCCCTGAGTT GGCGGTGGT-3′; *Foxa2* antisense: 5′-TTGCTCACGGAAGAGTAGCC-3′; *Rpl37a* sense: 5′-CAAGAAGGTCGGGATCGTCG-3′; *Rpl37a* antisense: 5′-ACCAGGCAAGTCTCAGGAGGTG-3′. Values of mRNA expression were normalized with the internal control gene *Rpl37a*. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Statistical analysis

Parametric data were presented as the mean ± standard error of the mean (SEM) and nonparametric data were presented as median with interquartile range. Comparisons were made with two-way ANOVA considering (i) time after birth and (ii) treatment during pregnancy. Tukey's multiple comparison test was used to indicate intragroup differences at different time points, and a Sidak multiple comparison test was used to indicate the differences between CTL and DEX at the same time points. When the comparison was made between CTL and DEX animals, Mann-Whitney U or Student's *t*-test was applied for nonparametric and parametric data, respectively. The normality of the data was checked using the Kolmogorov–Smirnov and Shapiro-Wilk tests (GraphPad Prism Software, Version 6.01, Inc., San Diego, CA, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Food and liquid intake of CTL or DEX-treated rats during late pregnancy

Food intake of CTL and DEX-treated pregnant rats was monitored from the 14th to the 19th day of pregnancy. No differences were found when comparing the mean daily food intake (mean ± SEM) of CTL (26.01 ± 0.88 g/day; n = 5) and DEX-treated (24.21 ± 1.46 g/day; n = 5) pregnant rats (P = 0.32). Mean daily liquid intake (mean ± SEM) of CTL (56.6 ± 6.5 ml/day; n = 5) was also similar to that of DEX-treated (49.2 ± 5.6 ml/day; n = 5) pregnant rats (P = 0.41).

3.2. Insulin, glucose and glucagon levels in rats born to DEX-treated mothers during early postnatal life

The highest serum insulin concentrations in pups born to CTL rats occurred at PND1 while in rats born to DEX-treated mothers this zenith was delayed to PND3. Consequently, serum insulin levels in rats born to DEX-treated mothers were reduced at PND1 (66% lower; $P < 0.001$) and increased at PND3 (89% higher; P = 0.0123) when compared to CTL. Serum insulin levels at PND21 were similar in both groups (Fig. 1A). Changes in blood glucose levels were also assessed. Pups born to CTL and DEX-treated mothers exhibited their highest blood glucose levels at PND3 and at PND21, respectively. Although similar at PND1 and at PND21, glucose levels of pups born to DEX-treated mothers at PND3 were significantly lower than those born to CTL mother (28% lower; $P < 0.0001$) (Fig. 1B).

Serum glucagon levels in rats born to DEX-treated mothers were similar to those of CTL at PND1 and PND3. However, rats exposed to DEX *in utero* experienced an increase in serum glucagon levels during lactation period so that their levels were increased at PND21 (16% higher than CTL; P = 0.025). In addition, intra-group comparisons revealed that serum glucagon levels of pups born to DEX-treated mothers were lower at PND3 than at PND21 (21% lower; $P < 0.001$) (Fig. 1C).

3.3. Pancreatic β-cell morphology in rats born to DEX-treated mothers during early postnatal life

Body weight of rats born to DEX-treated mother was lower than that of CTL at PND1 (36% lower; $P < 0.0001$) and remained reduced at PND3 and PND21 (respectively 54% and 25% lower than CTL; $P < 0.0001$). As for body weight, pancreatic weight increased during postnatal life in both CTL rats and rats born to DEX-treated mothers. However, the pancreas weight of rats born to DEX-treated mothers was lower than that of CTL at PND21 (13% lower; $P < 0.0001$). In opposition to pancreas weight, pancreatic islet area progressively decreased during postnatal life, reaching minimal levels at PND21 for both groups. Similarly, pancreatic islet area of rats born to DEX-treated mothers was lower than that of CTL at PND1 (22% lower; P = 0.007) (Table 1).

Pancreatic mass of β-cell progressively increased during the early postnatal period reaching maximal values at PND21 in both CTL and rats born to DEX-treated mothers (respectively 92% and 200% higher than PND1; $P < 0.05$). As the patterns of changes were similar in both groups, no differences at any particular moment of observation were detected (Fig. 2G). As observed for pancreatic β-cell mass, the percentage of β-cells in pancreatic islets exhibited a progressive increase throughout postnatal life, reaching the highest values at PND21 in both CTL and rats born to DEX-treated mothers (respectively 63% and 52% higher than PND1; $P < 0.0001$). At PND21, however, the percentage of β-cells in pancreatic islets was reduced in rats born to DEX-treated mothers (7% lower than CTL; P = 0.0028) (Fig. 2H). Pancreatic islet β-cell area in CTL rats presented a progressive reduction, reaching the lowest values at PND21 (54% lower than PND1; $P < 0.0001$). In rats born to DEX-treated mothers, instead, the pancreatic islet β-cell area remained constant during early postnatal life and lower than CTL at PND1 and PND3 (respectively 33% and 34% lower than CTL; $P < 0.05$ and $P < 0.0001$) (Fig. 2I). Representative images of pancreas sections immunostained for insulin are shown in Fig. 2A–F.

3.4. Pancreatic α-cell morphology in rats born to DEX-treated mothers during early postnatal life

In accordance to previously published data [20], we have found that α-cells are predominantly located at the periphery of the islets of pups born to both CTL and DEX-treated mothers. Our quantitative analysis revealed that pancreatic mass of α-cell in rats born to CTL rats exhibited an increase at PND3 and PND21 (62% and 77% higher than PND1; $P < 0.05$). On the other hand, pancreatic α-cell mass in rats born to DEX-treated mothers exhibited a progressive increase, reaching the highest values at PND21. At this age, pancreatic α-cell mass in rats born to DEX-treated mothers was higher than in CTL (112% higher; $P < 0.0001$) (Fig. 3G). The percentage of α-cells in pancreatic islets exhibited a progressive increase, reaching the highest values at PND21 in rats born to either CTL or DEX-treated mothers. At all ages evaluated in the present study, however, these values were higher in rats born to DEX-treated mothers than in CTL (17%, 10% and 15% at PND1, PND3 and PND21, respectively; $P < 0.01$) (Fig. 3H). The pancreatic islet α-cell area exhibited a progressive decrease, reaching the lowest values at PND21 in rats born to either CTL or DEX-treated mothers (approximately 50% lower than PND1; $P < 0.0001$). However, at all ages evaluated herein, the values were similar between the groups. (Fig. 3I). Representative images of pancreas sections immunostained for glucagon are shown in Fig. 3A–F.

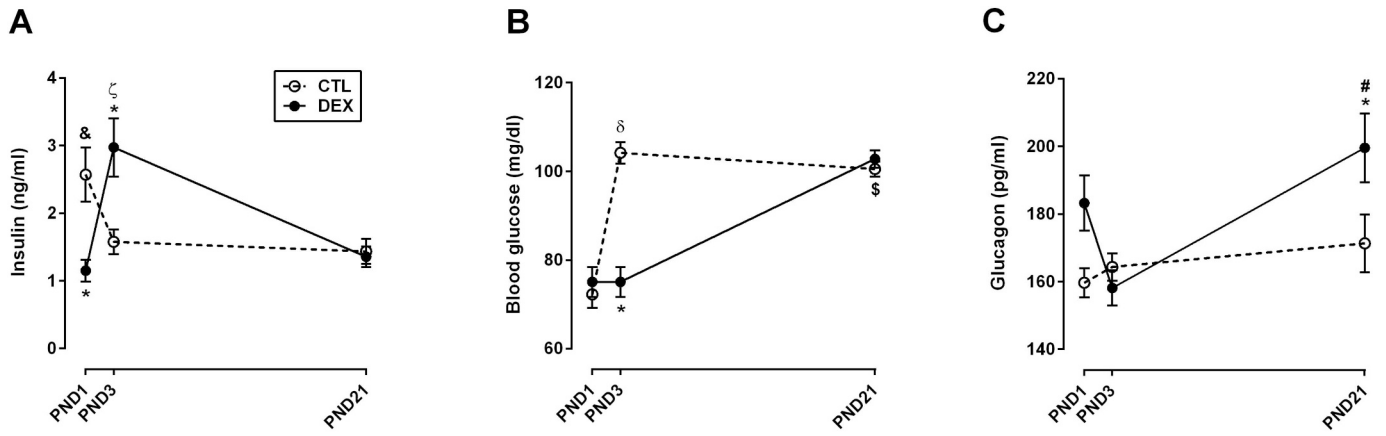

Fig. 1. Serum insulin (A), blood glucose (B) and serum glucagon (C) levels in the early postnatal life of offspring born to CTL and DEX-treated mothers during pregnancy. The rats were euthanized on the 1st (PND1), 3rd (PND3) and 21st (PND21) days after birth. Data had a parametric distribution and were presented as the mean ± SEM. Rats born to CTL mothers (n = 10–17) and rats born to DEX-treated mothers (n = 10–17) from at least 6 different mothers. Comparisons were made using two-way ANOVA. $^{*}P < 0.05$ *vs.* CTL at the same time points; $^{\delta}P < 0.05$ *vs.* CTL at PND1; $^{\&}P < 0.05$ *vs.* CTL at PND21; $^{\zeta}P < 0.05$ *vs.* DEX at PND1 and PND21; $^{\$}P < 0.05$ *vs.* DEX at PND1 and PND3; $^{\#}P < 0.05$ *vs.* DEX at PND3.

Table 1
Body weight and pancreas morpgerhometric data of offspring born to CTL and DEX-treated mothers.

	PND1		PND3		PN21	
	CTL	DEX	CTL	DEX	CTL	DEX
Body weight (g)	7.1 ± 0.05 (14)	4.5 ± 0.09 (12)*	10.1 ± 0.16 (16)	5.4 ± 0.27 (8)*	57.8 ± 0.74 (9)&	43.8 ± 1.97 (4)*,$
Pancreases weight (mg)	16.9 ± 0.08 (26)	20.1 ± 1.43 (34)	28.3 ± 1.24 (14)	20.8 ± 0.97 (17)	165.8 ± 9.72 (12) &	145.6 ± 7.20 (13)*,$
Islet area (μm²)	6025 ± 308	4744 ± 221*	4786 ± 236	4215 ± 213	2171 ± 160&	2051 ± 126$
Absolute number of islets analyzed[a]	556	688	1295	1841	647	874

The rats were euthanized on the 1st (PND1), 3rd (PND3) and 21st (PND21) days after birth. Parametric data are presented as the mean ± SEM. The numbers of independent measures are in parenthesis. Comparisons were made using two-way ANOVA.

* $P < 0.05$ *vs.* CTL at the same time points.

& $P < 0.05$ *vs.* CTL at PND1 and PND3.

$ $P < 0.05$ *vs.* DEX at PND1 and PND3.

[a] Represents the sum of the islets analyzed after immunostaining for insulin and glucagon in the pancreata sections.

3.5. Expression of genes related to pancreatic β-cell differentiation and function in rats born to DEX-treated mothers during early postnatal life

In addition to the morphological aspects of the pancreatic β-cells, we also assessed the expression of key genes that implicate in pancreatic β-cell secretory response and differentiation during the early postnatal life of rats born to DEX-treated mothers. The genes evaluated were *Ins2*, *Slc2a2*, *Gck*, *Ngn3*, *Neurod1*, *Pdx1, Pax4* and *Nkx6.1*. The expression of *Slc2a2, Gck*, *Neurod1* and *Nkx6.1* were increased in islets of rats born to DEX-treated mothers at PND1 (35%, 92%, 125% and 27% higher than CTL; $P < 0.05$) (Fig. 4A). The expression of *Ins2*, *Slc2a2*, *Neurod1* and *Pdx1* were increased in islets of rats born to DEX-treated mothers at PND3 (59%, 35%, 71% and 56% higher than CTL; $P < 0.05$) (Fig. 4B). The expression of *Slc2a2* was increased (70% higher than CTL; $P < 0.05$) while the expression of *Pax4* was reduced (33% Lower than CTL; $P < 0.05$) in pancreatic islets of rats born to DEX-treated mothers at PND21 (Fig. 4C).

3.6. Expression of genes related to pancreatic α-cell differentiation and function in rats born to DEX-treated mothers during early postnatal life

In parallel to the genes related to pancreatic β-cell phenotype, we also analyzed the expression of genes associated with pancreatic α-cell differentiation during early postnatal life of rats born to DEX-treated mothers. The genes evaluated were *Gcg*, *Pax6*, *Brn4*, *MafB*, *Nkx2.2* and *Foxa2*. The expression of *Gcg*, *Pax6*, *Brn4*, *MafB* and *Nkx2.2* were increased in pancreatic islets of rats born to DEX-treated mothers at PND1 (112%, 116%, 100%, 47%, 44% higher than CTL; $P < 0.05$) (Fig. 5A). The expression of *Gcg* and *MafB* remained increased in islets of rats born to DEX-treated mothers at PND3 (58% and 35% higher than CTL; $P < 0.05$) and at PND21 (62% and 48% higher than CTL; $P < 0.05$) (Fig. 5B and C, respectively).

4. Discussion

The present study systematically addresses the early postnatal morphological and transcriptional changes that occur in both pancreatic β- and α-cells of rats born to DEX-treated mothers. The information presently provided aid the comprehension of phenomena already described in clinical and experimental researches.

The pattern of serum insulin levels hallmarked by reduced values in rats born to DEX-treated mother at PND1 corroborates previous reports describing that exposure to DEX during fetal life impairs the formation of pancreatic β-cell [6]. We have found, however, similar glucose levels in pups born to CTL and DEX-treated mothers at PND1. Our interpretation for such data is that glucose levels in the newborn at PND1 might reflect the combined influence of the newborn insulinemia and maternal insulinemia at the end of pregnancy, which is known to be increased by DEX treatment [25].

Our experiments further revealed that the rats born to DEX-treated mothers exhibit an abrupt switch in serum insulin levels so that their values in PND3 become higher than those of CTL. The increased serum insulin levels in pups born to DEX-treated mothers at PND3 were concomitant with reduced blood glucose concentrations.

The present morphological data allows the conclusion that this rapid increase in insulin levels at PND3 seen in rats exposed to DEX *in*

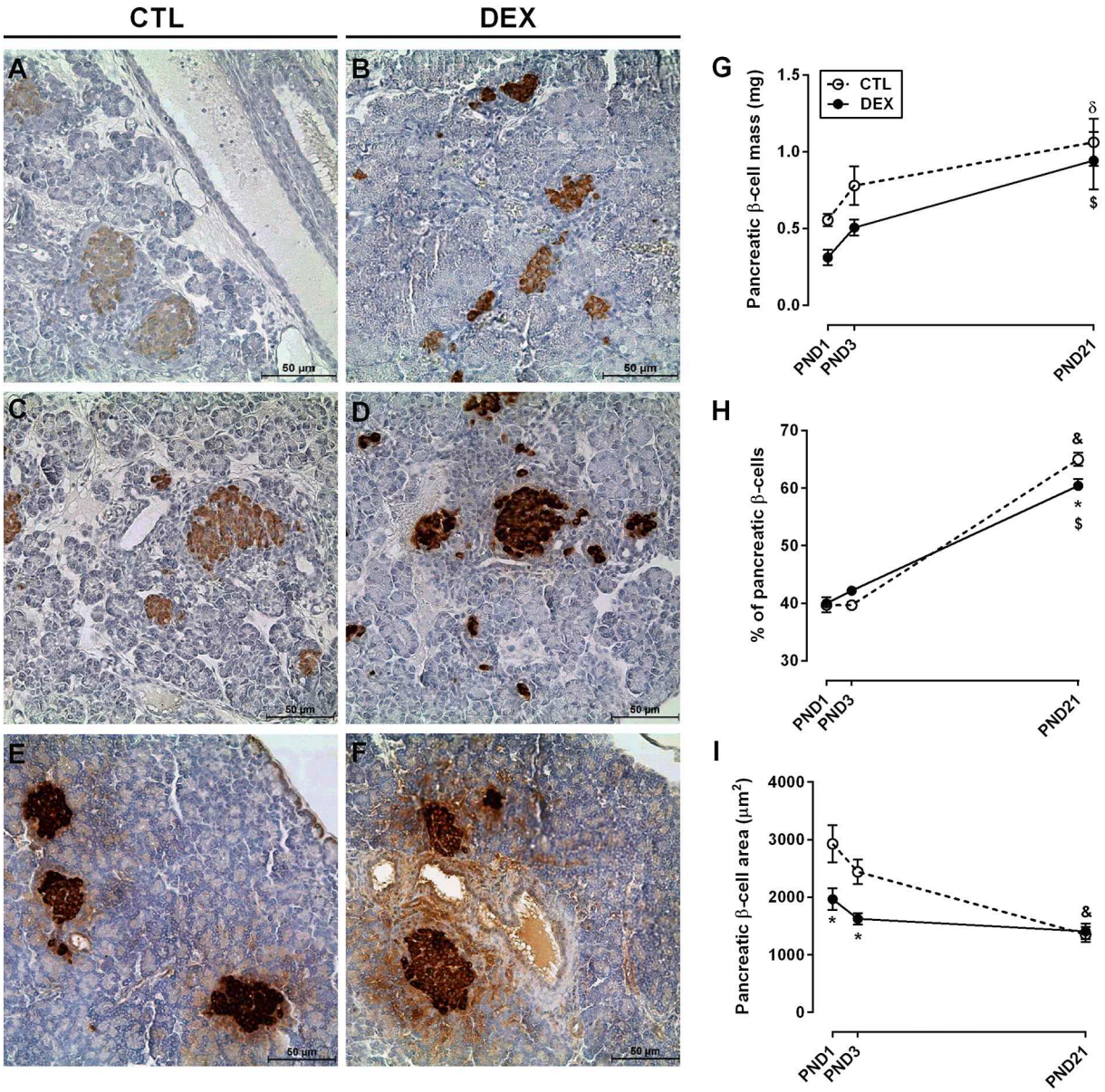

Fig. 2. *In utero* exposure to dexamethasone (DEX) regulates the pancreatic β-cell area and proportion, but not the β-cell mass, during the early postnatal life of the offspring. *Panels* show paraplast-embedded pancreas sections on the 1st (PND1) (A,B), 3rd (PND3) (C,D) and 21st (PND21) (E,F) days after birth, which were immunolabelled for insulin. Black bar represents a scale of 50 µm, objective 20×. Pancreatic β-cell mass (G), percentage of pancreatic β-cell (H) and pancreatic β-cell area (I) were analyzed in at least 8 pancreas sections per group (two sections per animal). Data had a parametric distribution and were presented as the mean ± SEM. Rats born to CTL mothers (n = 4) and rats born to DEX-treated mothers (n = 4). Comparisons were made using two-way ANOVA. $^{*}P < 0.05$ *vs.* CTL at the same time points; $^{\delta}P < 0.05$ *vs.* CTL at PND1; $^{\&}P < 0.05$ *vs.* CTL at PND1 and PND3; $^{\$}P < 0.05$ *vs.* DEX at PND1 and PND3.

utero is not due to a simultaneous increase in pancreatic β-cell mass and/or area, as these parameters remain lower than CTL at this moment of the postnatal life. On the other hand, our data reveal that islets isolated from rats born to DEX-treated mothers have increased expression of genes classically involved in insulin secretion such as *Slc2a2* and *Ins2* itself [26,27]. Thus, this data supports the notion that pancreatic β-cell of rats born to mothers treated with DEX present a delayed establishment of the insulin secretory response, without a parallel recovery of pancreatic β-cell mass.

This data also contributes to the interpretation of previously published findings. Somm and colleagues have previously demonstrated that insulin levels during a glucose tolerance test performed during the early postnatal life are higher in rats born to DEX-treated mothers than in CTL rats [19]. Our data showing the increase of genes related to insulin secretion and serum insulin levels at the early postnatal life with the concomitant reduction in blood glucose levels (PND3) in rats exposed to DEX *in utero* may also have a clinical relevance. A recent clinical trial evaluating >2800 women have demonstrated that late preterm babies born to mothers subjected to antenatal corticotherapy have a higher risk of hypoglycemia when compared to those born to mothers that did not receive the pharmacological intervention [28].

Other consequences of antenatal DEX treatment revealed by the present investigation is the increase in pancreatic α-cell mass as well as in its percentage within pancreatic islets during postnatal life. Information regarding α-cell morphology during postnatal life in this model were not available so far. We also describe that these morphological changes seen in rats born to DEX-treated mothers were paralleled by a persistent increase in *Gcg* expression and increased serum glucagon levels at PND1 and at PND21. In accordance to our data, increased serum glucagon levels and increased glucagon secretion have

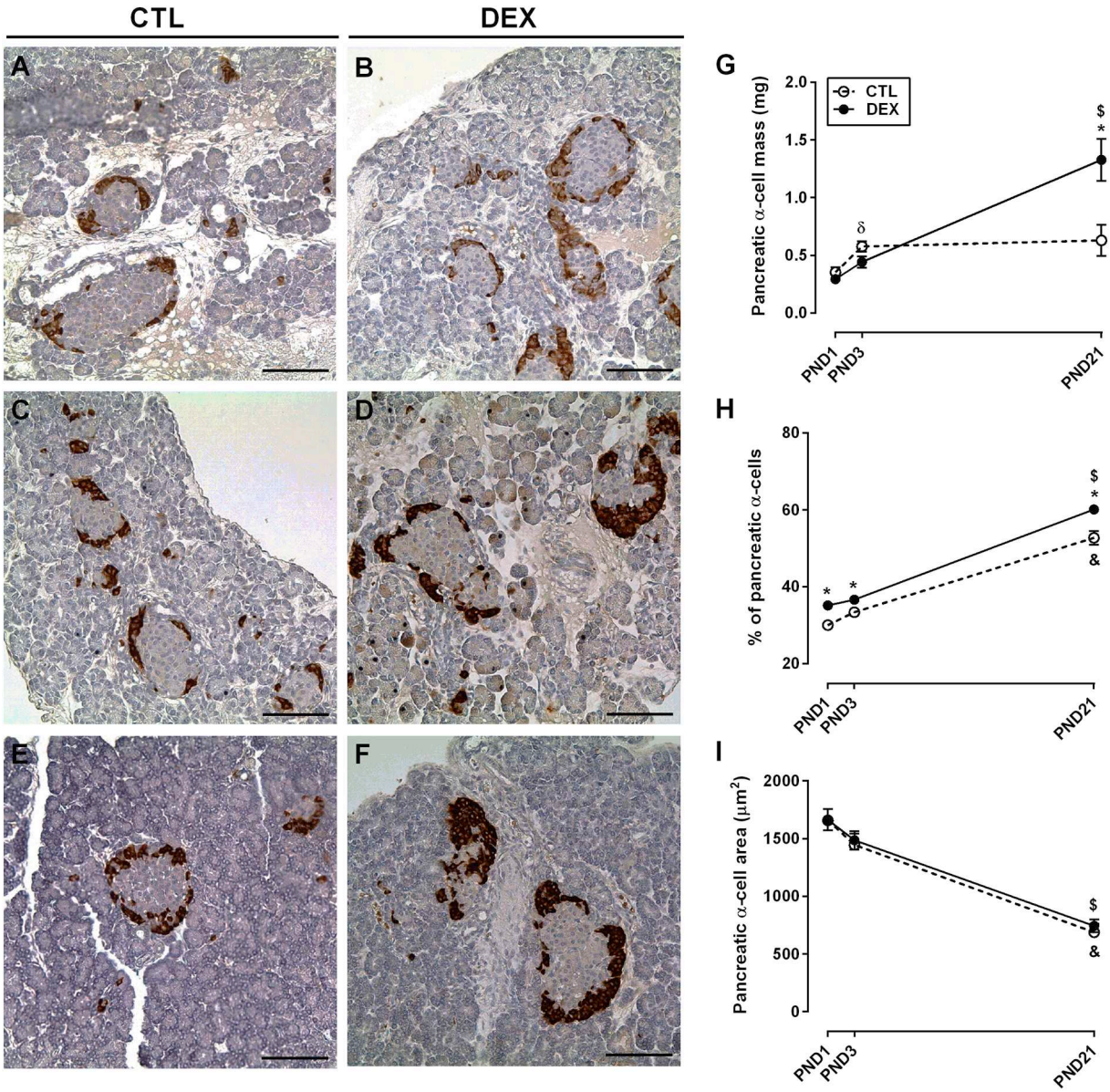

Fig. 3. *In utero* exposure to dexamethasone (DEX) regulates the pancreatic α-cell mass and proportion, but not the α-cell area, during the early postnatal life of the offspring. *Panels* show paraplast-embedded pancreas sections on the 1st (PND1) (A,B), 3rd (PND3) (C,D) and 21st (PND21) (E,F) days after birth, which were immunolabelled for glucagon. Black bar represents a scale of 50 μm, objective 20×. Pancreatic α-cell mass (G), percentage of pancreatic α-cell (H) and pancreatic α-cell area (I) were analyzed in at least 8 pancreas sections per group (two sections per animal). Data had a parametric distribution and were presented as the mean ± SEM. Rats born to CTL mothers (n = 4) and rats born to DEX-treated mothers (n = 4). Comparisons were made using two-way ANOVA. $^{*}P < 0.05$ *vs.* CTL at the same time points; $^{\delta}P < 0.05$ *vs.* CTL at PND1; $^{\&}P < 0.05$ *vs.* CTL at PND1 and PND3; $^{\$}P < 0.05$ *vs.* DEX at PND1 and PND3.

also been described in both fasted and fed rats subjected to DEX treatment during adult life [29].

This pattern of increased α-cell mass during postnatal life may also help to explain the already described upregulation of hepatic phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6pase) in the liver of rats born to DEX-treated mothers during postnatal life [9], as these genes are classically recognized as being stimulated by glucagon [30]. Our experiments also showed that rats exposed to DEX *in utero* exhibited a transitory reduction in serum glucagon levels at PND3 in spite of a permanent increase in its expression. As we understand, this reduction is due to an inhibitory action caused by the simultaneously increased insulin levels [31].

At the transcriptional level, our experiments show that rats born to DEX-treated mothers display increased expression of genes that stimulate the differentiation to common endocrine precursors and to α-cell. In this sense, the Pax6/MafB transcriptional factors were previously described to play a crucial role in stimulating glucagon expression and differentiation to α-cell independently of Foxa2. Such mechanism is likely to be more intense in situations of reduced Pax4 expression [32,33]. Accordingly, Pax4 was described to mediate differentiation of α- to β-cell [34], and its expression was found to be reduced in islets of rats born to DEX-treated mothers.

The increase in *Pdx1* and *Nkx6.1* expression, on the other hand, might contribute to the upregulation of *Ins2*, *Gck* and *Slc2a2* expression detected in islets of rats born to mothers treated with DEX, and thus contribute to their increased insulin levels [35–37]. *Pdx1* was described to act in multiple steps of pancreatic development, being required not only for maturation of the β-cell phenotype but also for the early expansion of common endocrine pancreatic precursor [38]. In agreement with our data and with these multiple roles of *Pdx1*, this transcription factor was already reported to be unable to repress glucagon expression in α-cell [39].

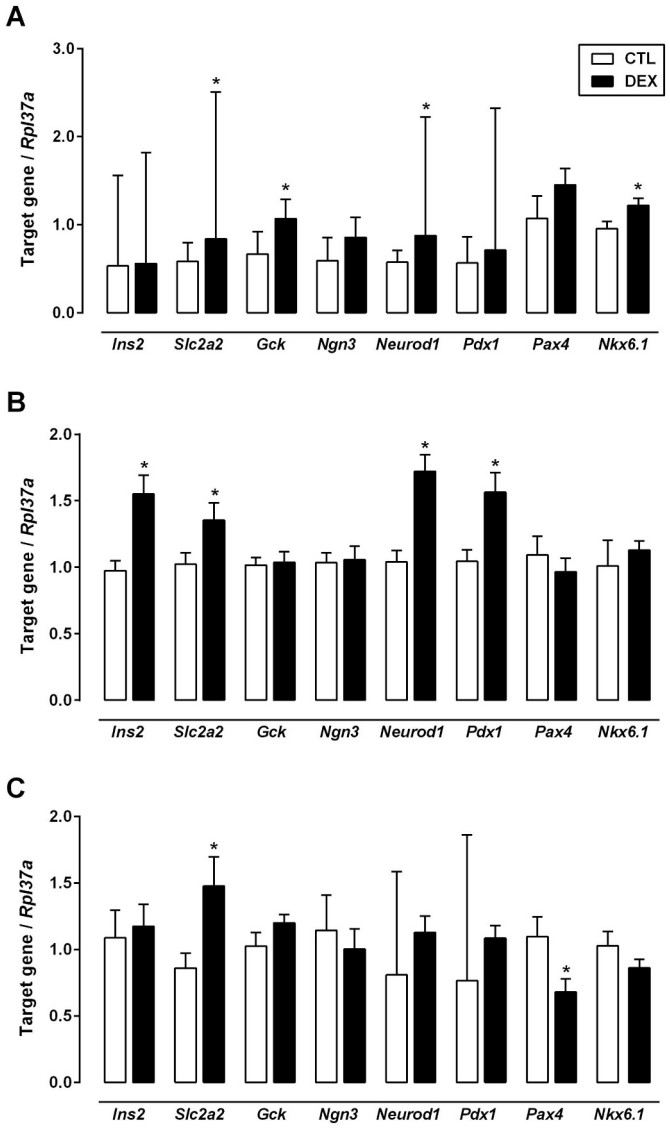

Fig. 4. mRNA expression of transcription factors related to β-cell function and development in the early postnatal life of offspring born to CTL and DEX-treated mothers during pregnancy. Pancreatic islets were isolated on the 1st (PND1) (A), 3rd (PND3) (B) and 21st (PND21) (C) days after birth, and processed for qPCR detection of *Ins2, Slc2a, Gck, Ngn3, Neurod1, Pdx1, Pax4* and *Nkx6.1*. Parametric data were presented as the mean ± SEM and nonparametric data were presented as median with interquartile range. Rats born to CTL mothers (n = 6–11) and rats born to DEX-treated mothers (n = 6–11) from at least 6 different mothers. Comparisons were made using Mann-Whitney U or Student's *t*-test. In panel A, all genes but *Pax4* and *Nkx6.1* had a nonparametric distribution. In panel B, all genes but *Nkx6.1* had a parametric distribution. In panel C, all genes but *Neurod1* and *Pdx1* had a parametric distribution. *P < 0.05 *vs.* CTL.

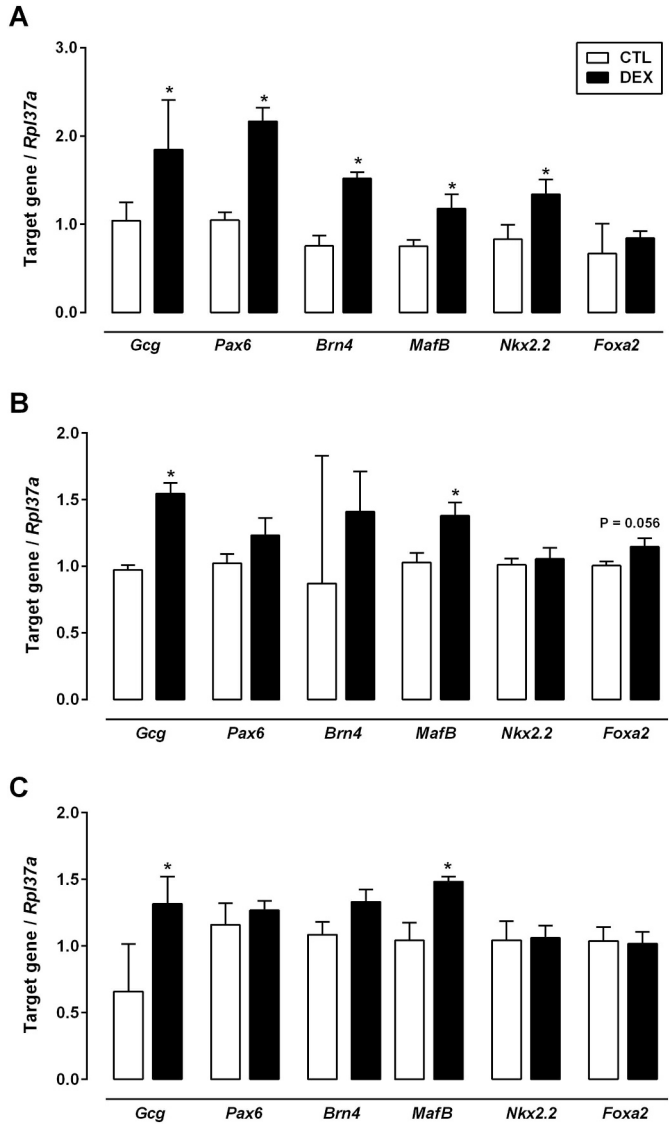

Fig. 5. mRNA expression of transcription factors related to α-cell function and development in the early postnatal life of offspring born to CTL and DEX-treated mothers during pregnancy. Pancreatic islets were isolated on the 1st (PND1) (A), 3rd (PND3) (B) and 21st (PND21) (C) days after birth, and processed for qPCR detection of *Gcg, Pax6, Brn4, MafB, Nkx2.2* and *Foxa2*. Parametric data were presented as the mean ± SEM and nonparametric data were presented as median with interquartile range. Rats born to CTL mothers (n = 6–11) and rats born to DEX-treated mothers (n = 6–11) from at least 6 different mothers. Comparisons were made using Mann-Whitney U or Student's *t*-test. In panel A, all genes but *Pax6* and *Brn4* had a nonparametric distribution. In panel B, all genes but *Brn4* had a parametric distribution. In panel C, all genes but *Gcg* had a parametric distribution. *P < 0.05 *vs.* CTL.

Other transcription factors that were increased in islets born to DEX-treated mothers during postnatal life, such as *Nkx2.2* and *Neurod1*, have been described to mediate the differentiation to both pancreatic β- and α-cell phenotype. For instance, it was previously shown that recovery of Nkx2.2 levels in Nkx2.2 knockout mice integrally rescue pancreatic α-cell mass and partially rescue β-cell mass [40]. NeuroD1, instead, stimulates the expression of prohormone convertase (PC2), the enzyme responsible for converting proglucagon in glucagon [41].

In summary, the present investigation shows that *in utero* exposure to DEX leads to postnatal changes in the morphology of the endocrine pancreas. These changes are hallmarked by increased α-cell mass and serum glucagon levels. Pancreatic β-cell mass is reduced during the postnatal period but an increase in serum insulin levels at PND3 is explained by upregulation of genes involved in insulin secretion. Transcriptional changes also comprise the upregulation of genes involved in the α- and β-cell phenotype.

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Fernando de Azevedo and Ivani Franco Correia dos Santos.Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.Author contributions

GFA, SB and JCS-S designed the research. JCS-S, PMRS, DNS and CJT performed the experiments and analyzed the data. SB and GFA contributed to the discussions and wrote the paper. All authors approved the final version of the manuscript.

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