Glucose homeostasis in rats treated with 4-vinylcyclohexene diepoxide is not worsened by dexamethasone treatment

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ABSTRACT

4-vinilcyclohexene diepoxide (4-VCD) causes premature ovarian failure and may result in estrogen deficiency, characterizing the transition to estropause in rodents (equivalent to menopause in women). Estropause/menopause is associated with metabolic derangements such as glucose intolerance and insulin resistance. Glucocorticoids (GCs) are known to exert diabetogenic effects. Thus, we aimed to investigate whether rats with premature ovarian failure are more prone to the diabetogenic effects of GC. For this, immature female rats received daily injections of 4-VCD [160 mg/kg body weight (b.w.), intraperitoneally (i.p.)] for 15 consecutive days, whereas control rats received vehicle. After 168 days of the completion of 4-VCD administration, rats were divided into 4 groups: CTL—received daily injections of saline (1 mL/kg, b.w., i.p.) for 5 days; DEX—received daily injections of dexamethasone (1 mg/kg, b.w., i. p.) for 5 days; VCD—treated as CTL group; VCD + DEX—treated as DEX group. Experiments and euthanasia occurred one day after the last dexamethasone injection.

4-VCD-treated rats exhibited ovary hypotrophy and reduced number of preantral follicles ($p < 0.05$). Premature ovarian failure had no impact on the body weight gain or food intake, but both were reduced by the effects of dexamethasone. The increase in blood glucose, plasma insulin and triacylglycerol levels as well as the reduction in insulin sensitivity caused by dexamethasone treatment was not exacerbated in the VCD + DEX group of rats. Premature ovarian failure did change neither the hepatic content of glycogen and triacylglycerol nor the glycerol release from perigonadal adipose tissue. Glucose intolerance was observed in the VCD group after an ipGTT ($p < 0.05$), but not after an oral glucose challenge. Glucose intolerance and compensatory pancreatic β-cell mass caused by GC were not modified by ovarian failure in the VCD + DEX group. We conclude that reduced ovarian function has no major implications on the diabetogenic effects promoted by GC treatment, indicating that other factors related to aging may make rats more vulnerable to GC side effects on glucose metabolism.

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1. Introduction

During natural aging many physiological functions may be impaired, *e.g.*, changes of neurologic, metabolic, and cardiovascular magnitudes. Almost all species have a lifespan that does not surpass their reproductive years [1]. This is not the case in women, who experience a reproductive senescence around the fifth decade of life as a consequence of a depletion of their finite pool of ovarian follicles, a process known as atresia [1]. This midlife transition state that culminates with reproductive senescence in women is termed perimenopause and can last 5–8 years on average until

menopause occurs (cessation of menstrual period) [2]. About one third of every woman's life is spent in the post-reproductive stage, since the average life expectancy at birth for both sexes worldwide is 71 years [3]. The impacts of perimenopause and menopause on health and diseases are unequivocally an important matter for investigation, which continuously supports the actions of the health-related professionals dealing with elderly patients.

Different animal models have been considered in scientific researches to mimic some aspects of women reproductive senescence, without the heterogeneous variations found in humans (e.g., genetic characteristics, diet, age, parity, socioeconomic status, and environmental exposures) [1]. Different from human females, rodents present an estrous cycle rather than a menstrual cycle and the estrous cycle lasts 4–5 days. Also, rodents do not exhibit menstruation, but have the uterine lining reabsorbed [4]. Rats and mice commonly experience irregular estrous cycle around 9–12 months of age, a condition termed estropause [1]. Three main laboratory models define the preclinical models for evaluation of reproductive aging: i) natural aging animals with intact ovaries (ovary-intact), ii) ovariectomized animals (OVX) and iii) a more recent model induced by the administration of 4-vinylcyclohexene diepoxide (4-VCD). Each model has its advantages and disadvantages (for a comprehensive review, read [1]).

4-VCD is a metabolite of 4-vinylcyclohexene, a compound formed from the dimerization of 1,3-butadiene in the manufacture of synthetic rubber, insecticides, plasticizers, flame retardants, and antioxidants [5]. It is also applied against pests, e.g., for reduction of rodent fecundity and proliferation [6]. 4-VCD selectively targets the small preantral ovarian follicles (mainly primordial and primary follicles), leading to an accelerated follicular depletion and different degrees of ovarian failure, depending on the dose and period of investigation after the completion of 4-VCD administration [7–10]. For instance, the complete interruption of cyclicity and the morphologic evidence for reproductive senescence (total absence of oocytes) occurs only around 350–360 days after 4-VCD treatment in rats [8]. Thus, 4-VCD-treated rodents become an interesting preclinical model for studying the transitional estropause. 4-VCD-treated rodents have the advantage of maintaining the ovarian follicles and circulating steroid hormones more similar to those found in women under menopause transition; aspects not mimicked by the ovary-intact or OVX rodent models [1].

The vast majority of studies conducted with 4-VCD model sought to elucidate its mechanisms of action and the comprehension of the profile of ovary-derived hormones (e.g., concentrations and time-course fluctuations) [7–15]. There are few studies exposing these animals to a physiological challenge [16–18]. For instance, 4-VCD-treated mice under menopause become more responsive to the angiotensin II hypertensive effects, which do not happen during perimenopause [18]. Similarly, mice become glucose intolerant 26 weeks after 4-VCD administration [17]. In both studies, 17β-estradiol replacement normalized the impairments. 17β-estradiol has important implications on the regulation of metabolism and there is a positive relationship between perimenopause/menopause and the development of metabolic-related derangements (for a comprehensive review, read Mauvais-Jarvis et al. [19]). These derangements include reduction of the energy expenditure, increased adiposity and altered adipocytokine secretory profile, β-cell dysfunction, reduced glucose disposal, which altogether may contribute to the development or exacerbation of obesity and/or type 2 diabetes mellitus [19]. Previous studies with 4-VCD-treated mice have not shown a significant alteration in the glucose metabolism (e.g., glycemia, insulinaemia and triacylglyceridemia) in normal animals [16,17]. However, the biological system may mask certain vulnerabilities while it is not

submitted to certain stressing or challenging conditions, e.g., exposure to diabetogenic hormones.

Glucocorticoids (GCs) are among the most effective diabetogenic hormones and there is substantial evidence with humans and rodents treated with synthetic GC (e.g., dexamethasone) in different contexts (acutely with high doses or chronically with low doses) demonstrating its impact on glucose and lipid metabolism such glucose intolerance, reduction of insulin sensitivity, elevation in blood glucose, plasma insulin and triacylglycerol levels, etc. (reviewed in [20–22]). We have a well-established model of rats treated with dexamethasone that exhibit prediabetes like phenotype (e.g., glucose intolerance, insulin resistance, elevation in blood glucose, plasma insulin, glucagon, tryacilglycerol levels) [23–25], which serves as a good model to stress the glucose homeostasis.

Thus, based on the evidence that 4-VCD administration in immature rats leads to ovarian failure, which may turn these animals vulnerable to metabolic insults [17], we sought to explore whether 4-VCD-treated rats are more susceptible or not to the well-known adverse effects of dexamethasone on glucose homeostasis during a period considered as estropause (168–173 days after completion of 4-VCD treatment). By treating Wistar rats with 4-VCD early in life and exposing them to a short-term high-dexamethasone treatment as adults, we were able to show that reduced ovarian function has no major implications on the diabetogenic effects promoted by GC treatment, indicating that other factors related to aging may turn rats more vulnerable to GC side effects on glucose metabolism.

2. Materials and methods

2.1. Ethical approval

The experiments with rats were approved by the Federal University of Santa Catarina Committee for Ethics in Animal Experimentation (approval ID: PP00782 and PP00842) that is in accordance with the National Council for Animal Experimentation Control (CONCEA).

2.2. Materials

4-Vinylcyclohexene diepoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone phosphate (Decadron®) was purchased from Aché (Campinas, SP, Brazil). Regular human recombinant insulin (Humulin®) was acquired from Lilly (Indianapolis, IN, USA). The reagents used in the glucose tolerance test, hepatic glycogen and fat content protocols, fat lipolysis protocol, and histology and immunochemistry were acquired from LabSynth (Diadema, SP, Brazil) and Sigma. Plasma insulin was quantified by AlphaLISA® technology (PerkinElmer, Waltham, MA, USA—cat. no. AL204). Plasma 17β-estradiol was determined by ELISA (DRG International, Inc., Springfield, NJ, USA—cat. no. EIA-2693). Plasma progesterone was determined using specific MP kits (MP Biomedicals; Orangeburg-NY, USA, cat. no. 07DE9988) [15]. Enzymatic colorimetric assay for the quantification of triacylglycerol, total cholesterol and lactate were from Biotécnica (Varginha, MG, Brazil—cat. no. BT1001000). Primary antibodies for insulin (sc-9168) and the ABC staining system (ImmunoCruz™) (sc-2018) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Animals

Experiments were performed on 3 groups of 40 female Wistar rats accounting for a total of 120 rats. The post-weaned rats were obtained from the Federal University of Santa Catarina Animal Breeding Center and were kept at $21 \pm 2\,°C$ on a 12-h light–dark cycle (lights on at 0600, lights off at 1800). Rats had access to food

From 28th to 42th days of age   From 210th to 214th days of age

2 GROUPS:

<u>CTL group</u> - treated with daily injection of corn oil (1.5 mL/kg, *b.w.*, *i.p.*)

<u>VCD group</u> - treated with daily injection of 4-VCD (160 mg/kg, *b.w.*, *i.p.*)

4 GROUPS:

<u>CTL and VCD groups</u> - treated with daily injection of saline (1.0 mL/kg, *b.w.*, *i.p.*)

<u>DEX and VCD+DEX groups</u> - treated with daily injection of dexamethasone (1.0 mg/kg, *b.w.*, *i.p.*)
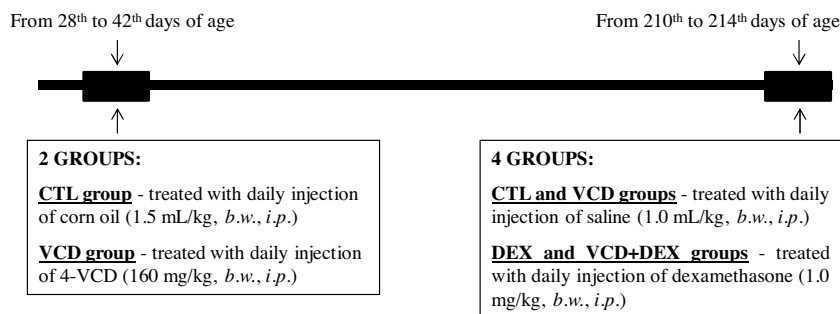
Fig. 1. Experimental design. At 28 days of age, female rats were separated into two groups and treated as detailed in left box for 15 consecutive days. When rats achieved 210 days of age (168 days after completion of 4-VCD or vehicle administration), they were divided into 4 groups and treated as detailed in right box for 5 consecutive days. For more detail, read Materials and Methods.

(commercial standard chow for rats, BIOBASE® 9301; Águas Frias, SC, Brazil) and water *ad libitum*.

2.4. 4-VCD and dexamethasone treatments

According to Fig. 1, post-weaned rats (21 days of age) were housed for acclimatization for 7 consecutive days and then were separated in two groups: VCD group—received a daily intraperitoneal (*i.p.*) injection of 4-VCD [160 mg/kg, body weight (*b.w.*)], between 0700 and 0800 h, for 15 consecutive days (from the 28th to 42nd days of age); CTL group—received a daily *i.p.* injection of corn oil (1.5 mL/kg, *b.w.*), between 0700 and 0800 h, for 15 consecutive days (from the 28th to 42nd days of age). These rats were used for evaluation of some behavior parameters (*e.g.*, ingestive responses in the open field and performance during an object recognition task) in a set of separated studies when they achieved around 90 days of life. Such behavioral experiments were not included in this study. None of the behavior parameters evaluated and animal handling is known to cause any kind of permanent stress in rats [26,27]. When rats achieved 210 days of age (168 days after completion of 4-VCD administration) they were divided into 4 groups: CTL group—received a daily *i.p.* injection of NaCl 0.9% (1 mL/kg, *b.w.*), between 0700 and 0800 h, for 5 consecutive days; DEX group—received a daily *i.p.* injection of dexamethasone phosphate (1 mg/kg, *b.w.*), between 0700 and 0800 h, for 5 consecutive days; VCD group—received a daily *i.p.* injection of NaCl 0.9% as CTL group, and VCD-DEX group—received a daily *i.p.* injection of dexamethasone as DEX group. The 4-VCD and dexamethasone dosing were based on previous publications [28–31]. Administration of 4-VCD during post-weaning window is known to render a state of early perimenopause and menopause when rats exceed around 60 and 120 days [8,15], which made this an useful experimental model for investigation of the effects of reduced ovarian function without aging as a confounding factor or the abrupt cessation of circulating ovarian hormones in

ovariectomized rats [1]. Dexamethasone at the dose and period administered is known to produce significant glucose metabolism side effects including glucose intolerance and reduction of insulin sensitivity, which make this a valuable tool for challenging glucose metabolism experimentally [23,24,30–32].

2.5. Metabolic measurements

Body weight was measured weekly, from 7 weeks before the beginning of dexamethasone administration, in order to check for 4-VCD treatment effects.Then, body weight and food intake were followed daily in the last 7 days before the euthanasia, which included the 5 days of dexamethasone administration. Fasted (12–14 h) rats had blood collected from the tail to measure blood glucose levels with a glucometer (Accu-Check® Performa; Roche Diagnostics GmbH, Mannhein, Germany). Immediately after blood collection, rats were euthanized (exposure to CO_2 followed by decapitation), and the trunk blood was collected in EDTA-NaF-containing tubes (Glistab—Labtest; Lagoa Santa, MG, Brazil) to obtain the plasma. The plasma, obtained after blood centrifugation (600g), was stored at −80 °C for posterior measure of plasma insulin, 17β-estradiol, progesterone, triacylglycerol, total cholesterol and lactate with the kits and methods described before according to the manufacturer's instructions. Organs (listed in Table 1) were gently withdrawn and weighed.

2.6. Determination of insulin sensitivity by the homeostatic model assessment (HOMA) and triacylglycerol and glucose index (TyG)

HOMA was calculated using the following formula: [fasting glycemia (mM) × fasting insulinemia (μU/mL)]/22.5 and TyG index was calculated using the following formula: Ln[fasting triacylglyceridemia (mg/dL) × fasting glycemia (mg/dL)/2] as previously published [32].

Table 1
Metabolic-associated and glucocorticoid-responsive organ mass (g/100 g *b.w.*) in CTL, VCD, DEX and VCD + DEX rats.

	CTL	VCD	DEX	VCD + DEX
Retroperitoneal[a]	1.90 ± 0.19	2.23 ± 0.12	2.07 ± 0.13	1.79 ± 0.15
Perigonadal[a]	1.19 ± 0.13	1.37 ± 0.09	1.39.5 ± 0.09	1.29 ± 0.12
Omental[a]	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.02	0.13 ± 0.01
Liver	2.59 ± 0.05	2.52 ± 0.05	4.13 ± 0.08***	3.99 ± 0.0.14**
Spleen	0.23 [0.201–0.25]	0.20 [0.19–0.22]	0.14 [0.16–0.16]***	0.14 [0.14–0.15]**
Adrenal[a,b]	30 [30–40]	30 [30–40]	25 [20–30]**	30 [27–30]

Values are means ± S.E.M or median [interquartile ranges]. $n = 7$–10. *Significantly different *vs.* their respective controls (CTL or VCD groups), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using ANOVA followed by Tukey *post test* (parametric data) or Kruskal-Wallis with Dunns *post test* (non-parametric data). When necessary parametric data were log transformed to achieve homocedasticity.

[a] Both bilateral organs were weighed.
[b] mg/100 g *b.w.*

2.7. Liver glycogen

Determination of hepatic glycogen content was performed according to previous publications with a few modifications [23,30]. Glycogen was determined by a phenol-based assay using a spectrophotometer. Determination of hepatic triacylglycerol content was in accordance with Gonçalves-Neto and colleagues [33]. Briefly, liver samples were transferred to test tubes containing 0.7 mL 1 M NaCl and homogenized with T18 UltraTurrax® (IKA®; Staufen, Germany). Then, methanol/chloroform solution (1:2 v/v) was added and the tubes were subsequently centrifuged. The methanolic phase was then transferred to another test tube and after wet the solution with in N_2 a solution of methanol/Triton 100 (1:2 v/v) was added to the samples for determination of hepatic triacylglycerol content that was normalized by the tissue fragment.

2.8. Intraperitoneal glucose tolerance test (ipGTT) and in vivo glucose-stimulated insulin secretion

Separate groups of fasted (12–14 h) rats were used for the ipGTT experiments. Conscious rats had their tail tip cut for blood collection. The first drop was discarded, and the second drop was used for the determination of glycemia (time 0) using a glucometer as described before. A 50% glucose solution pre-warmed at 36 °C (2 g/kg, *b.w.*, *i.p.*) was immediately administered, and blood samples were collected from the tail tip at 20, 40, 80 and 120 min for blood glucose measurements [23,30,31]. At minute 20, additional blood volume (60 μL) was collected in EDTA-NaF-containing tubes for plasma separation and posterior quantification of insulin as a measure of the *in vivo* glucose-stimulated insulin secretion [23,24].

2.9. Oral glucose challenging and in vivo glucose-stimulated insulin secretion

To evaluate the contribution of incretin glucagon-like peptide (GLP)-1 in the insulin response to the *in vivo* glucose stimulation, we performed a set of experiments in a separated group of rats. Rats were fasted and had glycemia (time 0) quantified as described before as for ipGTT. Immediately after, rats received an oral *bolus* of 50% glucose solution (2 g/kg, *b.w.*, oralgastric gavage) and 20 min after had their glycemia verified. Immediately, they were euthanized as described before for collection of trunk blood and determination of plasma insulin as a measure of the *in vivo* glucose-stimulated insulin secretion [24].

2.10. Adipose tissue lipolysis

This assay was performed with the same groups of rats used in Sections 2.5–2.7. After euthanasia the *ex vivo* adipose tissue lipolysis was assayed by incubating tissue samples and evaluating glycerol release into the incubation medium [32,34]. Perigonadal adipose tissue fragments (100 mg) were incubated in aerated (5% CO_2:95% O_2) Krebs buffer (pH 7.4) containing 1% bovine serum albumin for 60 min at 37 °C in the presence or absence of 20 μmol/L isoproterenol. At the end of the incubation, samples were collected and kept on ice. Glycerol levels were determined by an enzymatic colorimetric assay, similar to that described for plasma triacylglycerol.

2.11. Ovarian and pancreas tissue morphology and morphometry

To study morphological and morphometrical aspects of ovary and endocrine pancreas, 6 pars of ovaries and 6 pancreases (splenic fragment) from each group in a random selection were excised and processed according to a previous description [24,31] with the

exception that the fixative solution used for ovaries' fixation was 4% paraformaldehyde and for pancreases' fixation was 10% neutral buffered formalin. The organs were withdrawn from rats subjected to the oral glucose challenging. *Histology and immunostaining:* Ovary morphology was observed in hematoxylin & eosin stained sections. Pancreas sections were immunostained with insulin by the immunoperoxidase method and counterstained with hematoxylin according previous publication [24,31]. *Follicular ovarian morphometry:* For identification of 4-VCD effects on follicular development, three interspaced (250 μm) sections per ovary were cut and had preantral follicles and *Corpus luteum* counted, according to the classification described by Luo and collaborators [35]. Then, their relative masses (% of ovarian section) were multiplied by the ovarian weigh to obtain the absolute volume (in mm³) according to Juliani et al. [36]. *Beta-cell mass:* Beta-cell mass was determined by point counting morphometry on each pancreas section immunostained for insulin, according previous descriptions [24,31]. Each section was systematically scored with a grid of 120 points (final magnification ×200). An average of 250 fields per pancreas was counted. All the images for documentation were registered by a CCD camera coupled to a BX-60 Olympus photomicroscope.

2.12. Statistical analysis

The results are expressed as the mean ± SEM of the indicated number (n) of experiments. The symmetry of the data was tested by Kolmogorov-Smirnov and Shapiro-Wilk normality tests. Analysis of variance (ANOVA) (one-way ANOVA) for unpaired groups followed by Tukey's *post hoc* test were utilized for multiple comparisons of parametric data or Kruskal-Wallis followed by Dunn's *post hoc* when variables achieved asymmetric distribution. When indicated, data were log transformed for parametric analysis. ANOVA for repeated measures was applied when it was the case. Unpaired or paired Student's *t*-test or unpaired Mann-Whitney tests were applied when necessary. Extreme studentized deviate method was applied to determinate whether one of the values reached significant outlier (Grubb's test from online available GraphPad QuickCalcs). Reference in the text to "their respective control groups" means differences among DEX and VCD-DEX groups *vs.* CTL and VCD groups, respectively (effect of dexamethasone), or VCD and VCD-DEX groups *vs.* CTL and DEX groups, respectively (effect of 4-VCD). Significance was set at $p < 0.05$.

3. Results

3.1. Effects of 4-VCD treatment on ovaries

To confirm the biological effects of 4-VCD treatment early in life on the adult reproductive function we determined several reproductive-related parameters. The relative ovary weight (the sum of both glands), at the euthanasia, was significantly reduced in the 4-VCD-treated rats compared with their respective control groups (CTL and DEX) ($p < 0.01$; Fig. 2A). The values were 33.2 ± 1.7, 24.0 ± 1.0, 31.8 ± 1.0 and 24.6 ± 1.0 mg/100 g *b.w.* for the CTL, VCD, DEX and VCD + DEX groups, respectively. Plasma 17β-estradiol levels were ELISA-positive (nearing the lowest standard curve point of 25 pg/mL) in 4 from 10 samples (CTL group), none of 10 samples (VCD group), 1 from 10 samples (DEX group) and 1 from 10 samples (VCD + DEX groups). Plasma progesterone levels were not altered by 4-VCD treatment, but increased 18-fold in DEX *vs.* CTL groups ($p < 0.001$), an effect not observed in rats previously treated with 4-VCD (Fig. 2B). A panel of ovary tissue morphology emphasizing the reduction of preantral follicles and *Corpus luteum*
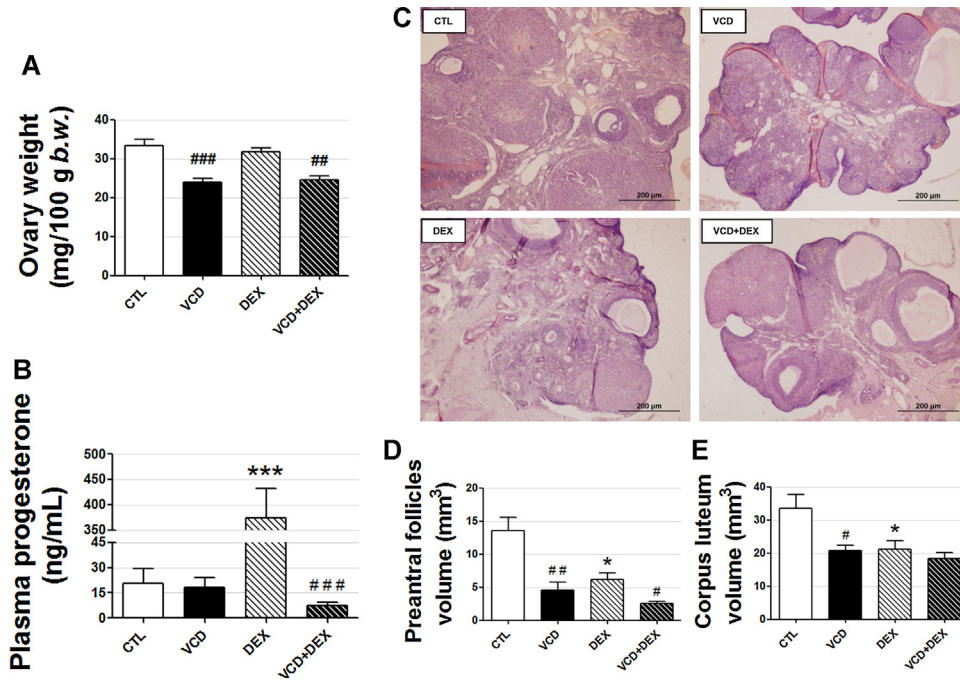
Fig. 2. 4-VCD treatment caused ovary hypotrophy and partial follicle depletion. Average ovary weight (A). Average fasting circulating progesterone (B). Histological longitudinal sections of ovaries in different experimental conditions stained by H&E (C). Average volume of preantral ovarian follicles and *Corpus luteum* (D and E). Data are presented as the mean $\pm$ SEM. $n = 6$ for A,C,D,E and 10 for B. *significantly different *vs.* the respective control group (effect of dexamethasone). #significantly different *vs.* the respective control group (effect of 4-VCD). *,#$P < 0.05$, **,##$P < 0.01$ and ***,###$P < 0.001$. ANOVA was applied followed by Tukey *post hoc* test. Data from A, D and E were log transformed before statistical analysis. Scale bar in C means 200 μm and was acquired through 4$\times$ objective.

distribution both in the 4-VCD-treated and dexamethasone-treated rats is observed in Fig. 2C ($p < 0.05$). The preantral and *Corpus luteum* volumes were quantified and the values can be observed in Figs. 2D and E. Based on that, we assume that 4-VCD treatment promoted ovary hypotrophy and reduced ovarian function in adulthood (173 days of age), which is compatible with the characteristics of estropause [8].

3.2. Murinometric and metabolic characteristics in the 4-VCD-treated rats

To verify whether 4-VCD treatment has any impact on murinometric and metabolic adverse effects caused by dexamethasone treatment, we evaluated several parameters. There were no differences in body weight between the CTL and VCD groups during the last 7 weeks before the beginning of dexamethasone treatment (Fig. 3A). During dexamethasone treatment, body weights were significantly reduced after 24 h and remained with a sustained reduction until euthanasia in DEX and VCD + DEX *vs.* CTL and VCD groups, respectively ($p < 0.01$). No differences in the final body weight were observed between the 4-VCD-treated rats and their respective controls (Fig. 3B and C). On the day before euthanasia, the body weight in the dexamethasone-treated rats was about 10% lower than in the control groups. Food intake was similar among the four groups before the initiation of dexamethasone treatment. Food intake was significantly lower in the dexamethasone-treated rats after 48 h of dexamethasone administration and remained lower until the day before euthanasia ($p < 0.01$; Fig. 3D and E). No alterations related to food intake were observed that could be attributed to the 4-VCD treatment. No modifications in blood glucose, plasma insulin, triacylglycerol, total cholesterol or lactate could be attributed to 4-VCD *per se* nor did the 4-VCD-treated rats become more vulnerable to the adverse

effects caused by dexamethasone treatment on such parameters (*e.g.*, elevation in glycemia, insulinaemia, triacylglyceridemia, lactatemia) (Fig. 4A–E). As judged by HOMA-IR and TyG indexes values, rats treated with 4-VCD did not show alteration in the insulin sensitivity (VCD group) neither did they show exacerbated insulin insensitivity caused by GC treatment (VCD + DEX group) (Fig. 4F and G). Neither 4-VCD nor dexamethasone treatment led to changes in rat adiposity (retroperitoneal, perigonadal and omental fat), but dexamethasone, as expected, caused adrenal and spleen hypotrophy as well as enhanced liver mass ($p < 0.01$) (Table 1). Altogether, reduced ovarian function caused by 4-VCD treatment had no impact on murinometric and metabolic profile *per se* nor worsened the adverse effects caused by GC treatment on murinometric and metabolic parameters.

3.3. Hepatic and adipose tissue function in the 4-VCD-treated rats

To verify whether 4-VCD treatment impact in the liver and fat function we measured the hepatic glycogen and triacylglycerol content and the glycerol released in the adipose tissue fragments. Exposure to 4-VCD early in life did not result in hepatic alterations (glycogen and triacylglycerol content) in adulthood neither did it caused additional impact when rats were treated with dexamethasone, a condition known to elevate both substrate contents (Fig. 5A and B). *Ex vivo* basal and isoproterenol-stimulated glycerol release was similar between CTL and VCD groups (Fig. 5C). The differences between stimulated *vs.* basal glycerol release were not observed in rats treated with dexamethasone (DEX and VCD + DEX groups) indicating a more lipolytic fat tissue at basal conditions. No differences between DEX and VCD + DEX were observed for this parameter. Thus, reduced ovarian function did not exacerbate the altered hepatic and fat functions caused by GC treatment.
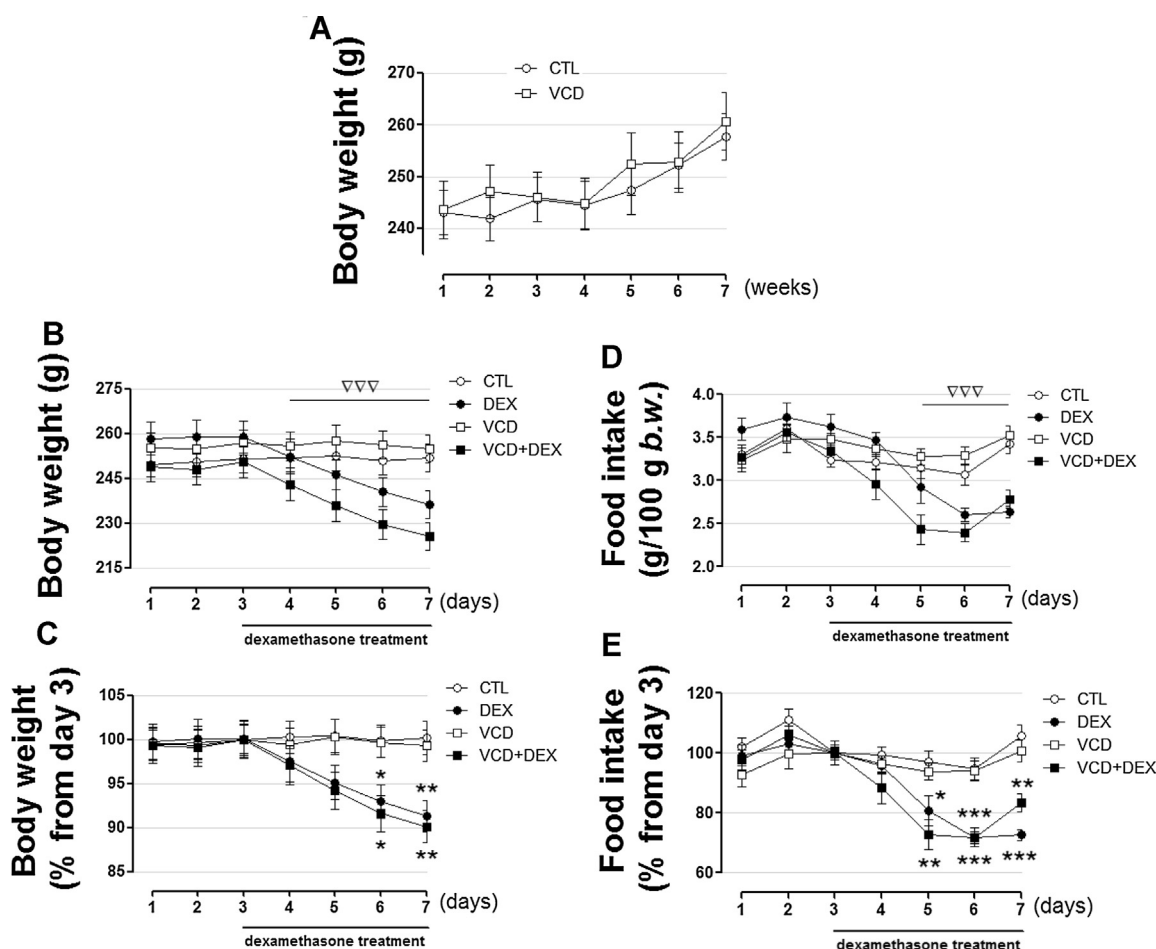
Fig. 3. 4-VCD treatment has no impact on body weight and food intake. Average body weight during the seven weeks preceding dexamethasone administration (A). Average body weight during dexamethasone administration until the day before euthanasia, expressed as absolute and relative data, respectively (B and C). Average food intake during dexamethasone administration until the day before euthanasia, expressed as absolute and relative data, respectively (D and E). Data are presented as the mean ± SEM. $n = 20$ for all graphs (ten rats from 2 different set of animals). *significantly different *vs.* the respective control group (effect of dexamethasone). $^{\triangledown}$significantly different from day 3. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\triangledown\triangledown\triangledown},^{***}P < 0.001$. ANOVA was applied followed by Tukey *post hoc* test (intergroup analyses) and ANOVA for repeated measures (intragroup analyses).

3.4. Glucose tolerance and in vivo glucose-stimulated insulin secretion in the 4-VCD-treated rats

Considering glucose intolerance may anticipate the elevation in blood glucose, we assessed the glucose tolerance and the insulin response to glucose *in vivo*. Rats treated with 4-VCD alone exhibited higher blood glucose levels 20 min and 40 min after an intraperitoneal glucose *bolus* than the CTL group ($p < 0.05$), indicating a negative impact of reduced ovarian function on glucose homeostasis. The blood glucose values were 372 ± 33 mg/dL for CTL group and 474 ± 19 mg/dL for VCD group at min 20, and 328 ± 23 mg/dL for CTL group and 435 ± 22 mg/dL for VCD group at min 40 (Fig. 6A). 4-VCD-treated rats that received GC treatment exhibited the same glycemic profile during the ipGTT test, which indicates no incremental impact of reduced ovarian function on glucose tolerance when rats receive GC. The area-under-curve of blood glucose values (A.U.C) revealed glucose intolerance in the VCD, DEX and VCD + DEX groups ($p < 0.05$; Fig. 6B). This glucose intolerance was associated to the reduced insulin response to glucose (at min 20 of ipGTT) in the VCD group ($p = 0.058$) and a relative deficiency (insufficient) of insulin secretion in those rats made insulin-insensitive due to the GC treatment (DEX and VCD + DEX groups) (Fig. 6C). The glucose intolerance was not

reproducible in VCD rats when challenged with glucose *bolus* by the oral vial, since they exhibited similar circulating insulin levels 20 min after glucose load (Fig. 6D and E). The VCD + DEX group did not exhibit additional alteration in the glucose profile or insulin secretion during the oral glucose challenge compared to the DEX group (Fig. 6D and E). Although no major impact on glucose tolerance by oral challenge was observed in VCD-treated rats, caution is required when combining the GC therapy with a reduced ovarian function.

3.5. β-cell mass in the 4-VCD-treated rats

To verify if a reduction in the ovarian function affects the compensatory increase in the β-cell mass in response to the diabetogenic actions of GCs, we evaluated this pancreas parameter. Treatment with 4-VCD early in life did not result in distinguished alteration of the β-cell mass in adulthood (Fig. 7A–C). When these 4-VCD-treated rats received dexamethasone during adulthood there was no equivalent increase in the β-cell mass as observed in the DEX group. The VCD-DEX group had reduced β-cell mass compared to the DEX group ($p < 0.05$; Fig. 7A and B), but after normalization by the body weight, the relative β-cell mass was higher in VCD + DEX group *vs.* VCD group ($p < 0.05$; Fig. 7C). Thus,
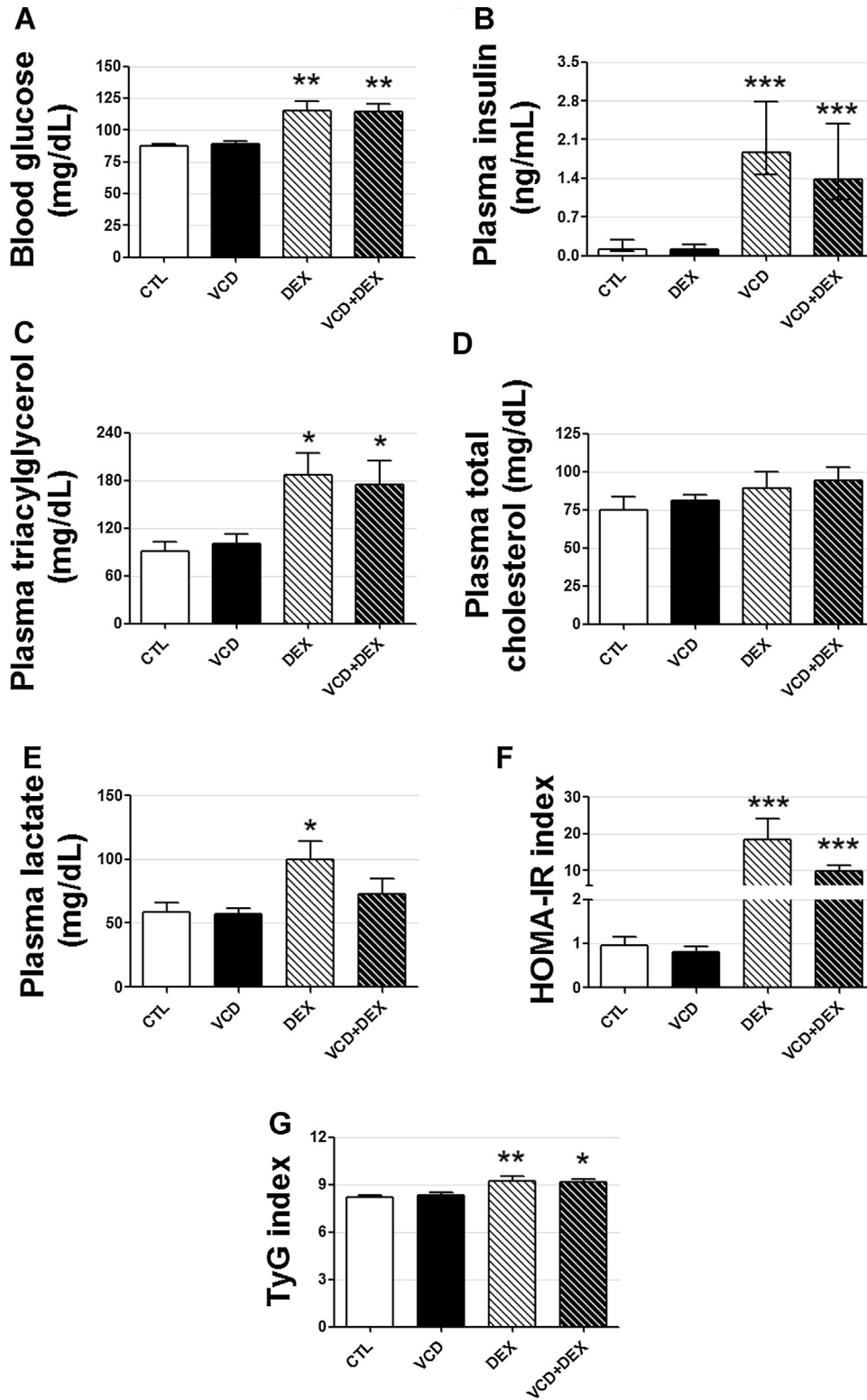
Fig. 4. Rats treated with 4-VCD exhibit no incremental change in metabolic parameters and insulin sensitivity caused by dexamethasone treatment. Average fasted blood glucose (A), plasma insulin (B), triacylglycerol (C), total cholesterol (D), lactate (E), HOMA-IR index (F), and TyG index (G). Data are presented as the mean ± SEM in A,C–F and median with interquartile ranges in B. $n = 10$ for all graphs. *significantly different vs. the respective control group (effect of dexamethasone). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. ANOVA was applied followed by Tukey post hoc test in parametric data and Kruskal-Wallis followed by Dunn's post hoc test in nonparametric data. Data from E were log transformed before statistical analysis.
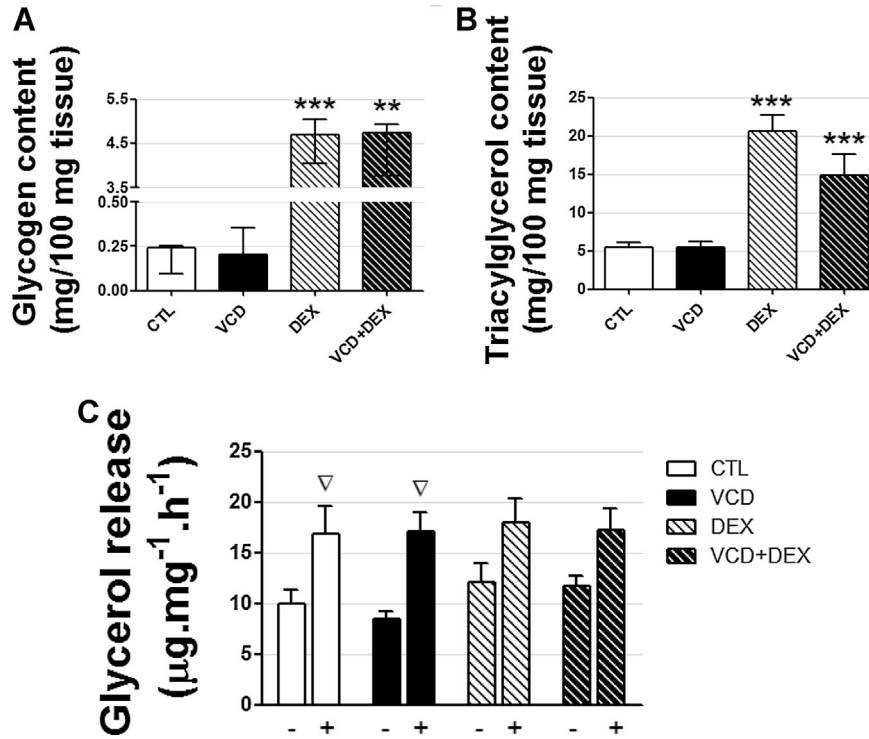
Fig. 5. Accumulation of hepatic glycogen and triacylglycerol caused by dexamethasone treatment is not worsened in rats with ovarian failure. Average hepatic glycogen (A) and triacylglycerol (B) content. In (C) average perigonadal glycerol release under basal and isoproterenol-stimulated conditions. Data are presented as the median with interquartile ranges in A and mean ± SEM in B and C. $n = 10$ for all graphs. *significantly different vs. the respective control group (effect of dexamethasone). $^{\nabla}$significantly different from basal condition. $^{\nabla}P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. ANOVA was applied followed by Tukey post hoc test in parametric data and Kruskal-Wallis followed by Dunn's post hoc test. Unpaired Student's t-test was also applied in C for comparison between stimulated and nonstimulated glycerol release. Data from B were log transformed before statistical analysis.
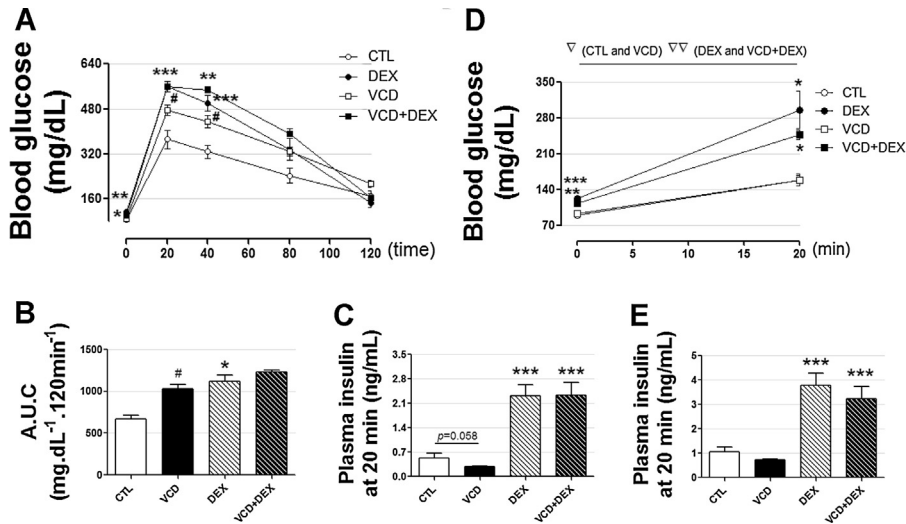

Fig. 6. Glucose intolerance caused by dexamethasone treatment is not exacerbated in rats with ovarian failure. Average blood glucose values during an intraperitoneal glucose tolerance test (2 g/kg, b.w.) (A) and the respective area-under-curve (B). Glucose intolerance observed in VCD group was paralleled with a nonsignificant reduction in insulin response to glucose in vivo 20 min post-glucose load (C). Average blood glucose values during a challenge with an oral bolus of glucose (2 g/kg, b.w.) (D). The average plasma insulin values at min 20 post-glucose load (E). Data are presented as mean ± SEM. $n = 10$ for all graphs. *significantly different vs. the respective control group (effect of dexamethasone). #significantly different vs. the respective control group (effect of 4-VCD). $^{\nabla}$significantly different from minute 0. $^{\nabla,*,\#}P < 0.05$, $^{\nabla\nabla,**}P < 0.01$ and ***$P < 0.001$. ANOVA was applied followed by Tukey post hoc test in parametric data. Unpaired Student's t-test was also applied in C for comparison between CTL and VCD groups. Data from C and E were log transformed before statistical analysis.
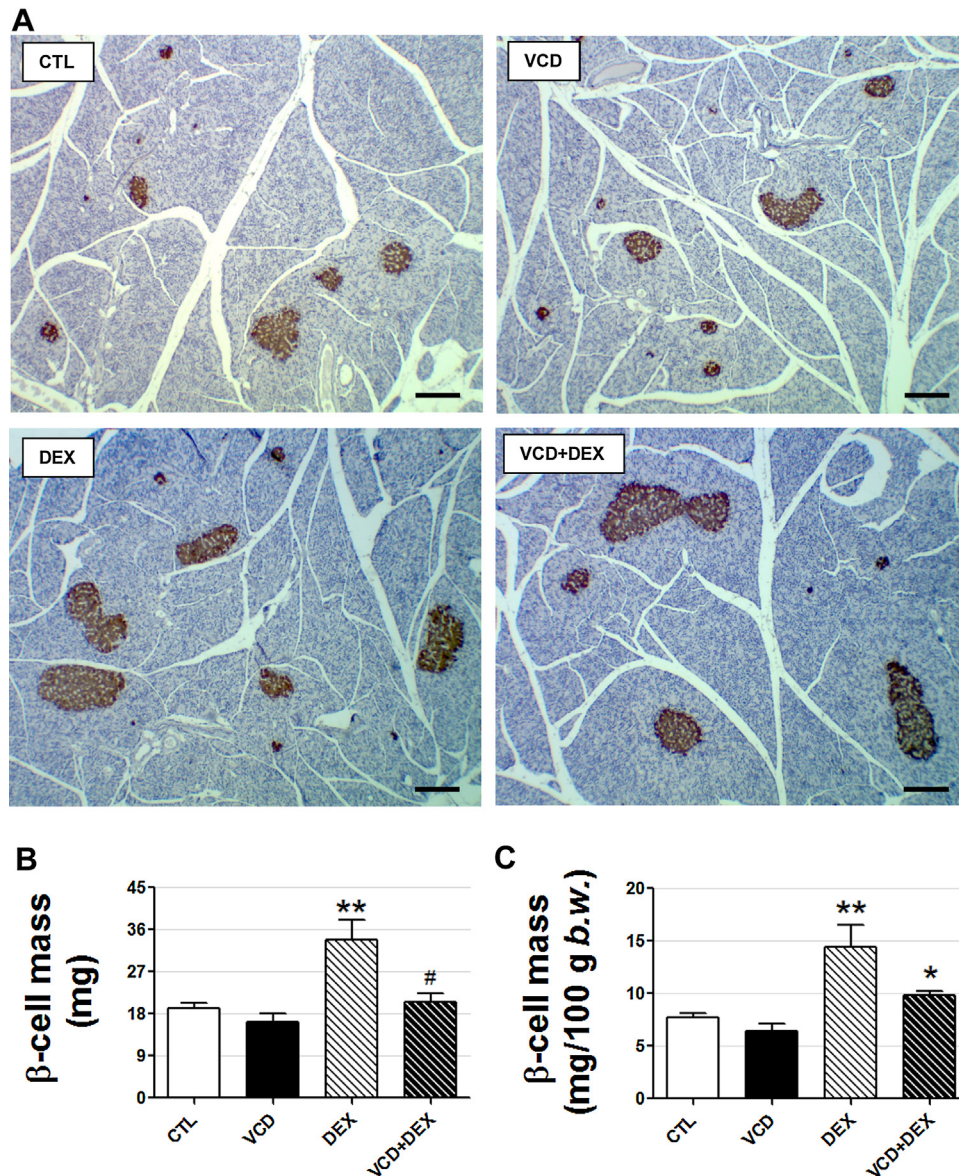
Fig. 7. The compensatory increase in pancreatic β-cell mass caused by dexamethasone treatment is partially attenuated in rats with ovarian failure. In (A) panoramic view of pancreas sections in the four groups. The average absolute (B) and normalized β-cell mass (C). Data are presented as the mean ± SEM. $n = 6$ for all graphs. *significantly different vs. the respective control group (effect of dexamethasone). #significantly different vs. the respective control group (effect of 4-VCD). *,#$P < 0.05$ and **$P < 0.01$. ANOVA was applied followed by Tukey post hoc test. Data from B and C were log transformed before statistical analysis. Scale bar in A means 200 μm and images was acquired with 4× objective.

failure of ovarian function per se had no implication in the β-cell mass, but impacted on the proper endocrine pancreas ability to increase β-cell mass in a diabetogenic context.

4. Discussion

Estrogens play an important role in the control of energy balance and glucose homeostasis [19]. Women and laboratory rodents experience a progressive transition toward a reduction in the ovary function during the post-reproductive period of life, which culminates in an altered steroid hormone profile (e.g., decrease in 17β-estradiol levels in women and variable circulating 17β-estradiol oscillations in rodents) [1]. Reduced estrogen plasma levels and/or actions may predispose the organism to many metabolic derangements (e.g., increase in visceral adiposity, glucose intolerance and peripheral insulin resistance) [19]. Here, we have demonstrated for the first time that adult rats with

premature ovarian failure (e.g., ovary hypotrophy and reduced preantral follicles volume), caused by 4-VCD administration early in life, exhibit the same degree of GC side effects on murinometric and glucose metabolism parameters: i) loss of body weight in parallel with hypophagia, ii) augmentation in blood glucose, plasma insulin and triacylglycerol levels, iii) insulin resistance, iv) increase in hepatic triacylglycerol content, v) glucose intolerance accompanied by relative poor insulin secretory response to glucose and vi) compensatory increase in pancreatic β-cell mass (to a lesser extend in VCD + DEX group).

The effectiveness of 4-VCD administration in the VCD and VCD + DEX groups was consistent with previous studies demonstrating a reduction in the ovary weight 30 days after the completion of 4-VCD administration in adult rats (80 mg/kg, b. w., i.p.) [37,38]. Both studies have demonstrated a decrease in circulating 17β-estradiol and an increase in follicle-stimulating hormone (FSH) 30 days after 4-VCD treatment. The dose of 4-VCD

administration and the time post-treatment evaluated in these studies were quite different from ours [37,38], which could explain the different results. The study conducted by Reis et al. [15] considered the periestropause period (analogous to perimenopause in women) around to 80 days after the beginning of 4-VCD treatment. Although we did not evaluate circulating FSH levels in our study (rats with 173 days of life after the beginning of 4-VCD treatment), other studies have demonstrated an increase in plasma FSH levels in rats (80 mg/kg, *b.w.*, *i.p.*) and in mice (160 mg/kg, *b.w.*, *i.p.*) 120 days after the onset of 4-VCD administration [8,28], which is consistent with estropause (analogous to menopause in women). Only in the CTL group, 4 out of 10 plasma samples were positive for 17β-estradiol in the ELISA experiments; a frequency three to four times higher compared to the other three groups. This result are in accordance with a previous study showing that mice treated with 4-VCD (160 mg/kg *b.w.*) early in life also showed nondetectable levels of 17β-estradiol on day 127 following the onset of 4-VCD treatment [28]. This indicates that both 4-VCD and dexamethasone treatments had a negative impact on 17β-estradiol bioavailability. The lack of estrous cycle information in our rats is a drawback of our study, but we believe the CTL group was regularly cycling, which might explain why this group presented a higher frequency of 17β-estradiol positive tests. These is corroborated by the knowledge that estrous cycle duration becomes longer in 4-VCD-treated rodents, including in mice treated with similar conditions to our model (known as more sensitive to 4-VCD) [29]. The effectiveness of ovarian failure in the 4-VCD-treated rats was also in accordance with previous studies revealing a reduction in the ovarian number of preantral follicles and *Corpus luteum* after 4-VCD treatment [8–10,13,39]. It is important to highlight that 4-VCD treatment does not eliminate all follicles. In fact, 4-VCD-treated rats, submitted to the same protocol used in our study, present the same amount of healthy follicles, but an increased number of atresic follicles [15]. Data with circulating progesterone in the present study also indicate an ovarian failure in the 4-VCD-treated rats as the GC-induced elevation in plasma progesterone levels was abolished in the VCD + DEX group. This elevation in plasma progesterone levels in the DEX group may be a result of direct effects of GCs on progesterone biosynthesis and release from the ovaries [40,41].

Considering estrogens are involved with modulation of energy balance [19] we accompanied the body weight gain along animal's development. Ovarian failure had no significant impact on body weight gain before and during dexamethasone treatment. The significant reduction in body weight was associated to dexamethasone treatment and could be explained, at least in part, by the parallel reduction of food intake along with the GC treatment. Rats treated with dexamethasone have exhibited hyperinsulinemia [24,25,32,34,42,43] and hyperleptinemia [42]. Both of which are signals to activate satiety in the hypothalamic nuclei involved in the control of appetite. There is also evidence for a negative water balance in rats treated with dexamethasone. This might contribute to body weight reduction [44]. Whether 17β-estradiol hormone had a major implication on body weight in the 4-VCD-treated rats, then an opposite scenario of augmented food intake would be expected in the VCD and VCD + DEX groups. Although there is evidence for an anorexic role of 17β-estradiol [19,45], that was not the case in the present study.

Short-term treatment with dexamethasone is a valuable tool to stress and challenge the glucose homeostasis once GCs exert diabetogenic effects (*e.g.*, hyperglycemia, hyperinsulinemia, hyperglucagonemia, hypertriacylglyceridemia, insulin resistance) in both human and rodents [20–22]. All of these metabolic alterations, except for hyperglucagonemia (not quantified in the current study), were confirmed in the dexamethasone-treated rats (DEX) while rats with reduced ovary function (VCD + DEX) did not

exhibit exacerbation of such parameters. Rats treated with 4-VCD alone did not reveal any alteration on these parameters. This absence of 4-VCD impact on metabolic parameters was in accordance with previous publications showing that mice treated with 4-VCD had no alteration in blood glucose values when evaluated both during periestropause and estropause [16,17]; neither of these two studies measured circulating 17β-estradiol. An association of a high-fat diet with ovarian failure likewise did not result in a worsening of blood glucose and plasma triacylglycerol values in mice during estropause [17]. Dexamethasone-mediated actions in such metabolic alterations include increase in hepatic gluconeogenesis [21,25,34], reduced adipose tissue and skeletal muscle glucose uptake [21,46], hyperglucagonemia [21,25], reduced insulin signaling in peripheral tissues [34,46], increased adipose tissue glycerol release [32,34,46] and reduced insulin-degrading enzyme activity in liver [47]. Although the downregulation of circulating estrogen levels or its peripheral actions impact negatively in the glucose and lipid homeostasis [19,45], it seems not to be the case in the current 4-VCD rat model. It indicates that residual circulating 17β-estradiol still exerts effects and/or other components related to the aging process might implicate the estrogen downregulation on metabolic-related derangements occurring in the reproductive senescence. The mechanism by which estrogens act in this context remains unclear, but it has been proposed that the age-related shift in estrogens efficacy could be related to an alternative splicing of estrogen receptors in rats [48].

Evaluation of glycogen and triacylglycerol hepatic contents revealed no negative implication of 4-VCD administration *per se* or when associated with the dexamethasone challenge. Both groups receiving 4-VCD early in life presented the same hepatic content values compared to their control groups. The enhanced hepatic glycogen and triacylglycerol content in the dexamethasone-treated groups are partially explained by an alleviation of glycogen consumption due to an increased hepatic gluconeogenic action of GC [21,25,34] and increased hepatic lipid biosynthesis (*e.g.*, by stimulating the activity of acetyl-coenzyme A carboxylase and free fatty acid synthase, increasing the synthesis of VLDL, down-regulating LDL receptor activity and increasing the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase) [49]. 4-VCD treatment exerted no major effects on the glycerol release from the perigonadal adipose tissue which was in accordance to their absent effect on the circulating triacylglycerol levels. The absence of significant differences between basal and isoproterenol-stimulated glycerol release in rats treated with dexamethasone is in agreement with previous studies showing similar results [34,50] and may be associated to reduced lipoprotein lipase activity [51] and augmented hormone-sensitive lipase activity [52].

Considering 4-VCD treatment promoted no major impact on basal glucose and lipid homeostasis, we challenged the rats' glucose homeostasis with an intraperitoneal glucose tolerance test (ipGTT), a method for evaluation of insulin secretory capacity (without contribution of incretin hormones), peripheral insulin sensitivity and glucose effectiveness [53]. The VCD group became glucose intolerant as observed by AUC values, obtained during ipGTT, and it was associated with a reduced insulin secretory response to *in vivo* glucose stimulation. However, no alteration in blood glucose levels was observed after a 20 min challenge with an oral *bolus* of glucose, which was paralleled with a normal insulin secretory response *in vivo*. Treatment with 4-VCD did not worsen the glucose intolerance induced by dexamethasone administration. Thus, excluding the incretin contribution to the potentiation of glucose-stimulated insulin secretion, the VCD group seemed to have an impairment of the secretory process. This is in accordance to the evidence that 17β-estradiol, through estrogen receptor β, augment glucose-stimulated insulin secretion [54]. In agreement

with that, estropaused mice exhibit glucose intolerance 24 weeks after 4-VCD treatment when submitted to an ipGTT. However, this is normalized after 17β-estradiol replacement [17], showing the consequence of estrogens deficiency on glucose homeostasis. Santos et al. [31] have demonstrated that 12-month old female rats become insulin resistant and show reduced islet insulin secretory response to glucose. Although these authors did not measure circulating estrogens, they suggested that steroid hormones may be involved with the reduced islet response to glucose.

The VCD + DEX group responded to the ipGTT and oral challenge with a *bolus* of glucose in a similar way to the DEX group. This resembling response may be explained by their capacity to increase the pancreatic β-cell mass (when normalized by their body weight) in response to the reduced peripheral insulin sensitivity. Although the magnitude of this adaptive islet compensation in VCD + DEX group was different from the DEX group, it was enough to avoid the exacerbation of glucose intolerance caused by GC administration. Estrogens are implicated in the maintenance of pancreatic β-cell formation and replication during metabolic injury [55]. Thus, we cannot discard that VCD + DEX group retained residual levels of estrogen, enough to play a role on the relative compensatory increase in the β-cell mass. Moreover, they could have exhibited an upregulation of estrogen receptors or signaling in the pancreatic β cells. Indeed, estrogens are important to the physiological maintenance of β-cell mass. Ovariectomized rats exhibit reduction in the β-cell mass and this impairment is recovered by 17β-estradiol replacement [45].

Altogether, these results demonstrated a reduction in the ovarian function based on ovary hypotrophy, reduced number of preantral follicle and *Corpus luteum*, and abolishment of progesterone elevation induced by GC. The adverse effects of dexamethasone on glucose metabolism (*e.g.*, glucose intolerance, insulin resistance, dyslipidemia) were not worsened by the treatment with 4-VCD early in life. Therefore, we conclude that premature ovarian failure in adult rats does not exacerbate the negative impact of GC on glucose and lipid metabolism. These data suggest that there is a residual action of estrogens in the 4-VCD model for rats and other components present during aging might act conjunctly with estrogen deficiency to cause the metabolic derangements associated with estropause in rats.

Conflict of interest

The authors declare no conflict of interest.

Authorship contributions

Participated in research design: A.R. and F.B.L. Conducted experiments: A.M.B., A.R., C.S, E.C.R.L., F.G.B, S.R.T. and S.S. Contributed with analytic tools and data analysis: A.R., F.B.L. and F.G.B. Performed data collection and interpretation: A.R., F.B.L. and F.G.B. Provided reagents/materials: A.R., F.B.G. and J.A.A-F. Wrote the paper: A.R. Contributed to discussion: A.R. and F.B.L. All authors read and approved manuscript final format.

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