

The New Zealand obese mouse model of obesity insulin resistance and poor breeding performance: evaluation of ovarian structure and function

Simone Radavelli-Bagatini¹, Amy R Blair, Joseph Proietto, Poli Mara Spritzer^{1,2} and Sofianos Andrikopoulos

Department of Medicine (Austin Health/Northern Health), Heidelberg Repatriation Hospital, The University of Melbourne, 300 Waterdale Road, Heidelberg Heights, Melbourne, Victoria 3081, Australia

¹Department of Physiology, Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Rio Grande do Sul 90035-003, Brazil

²Gynecological Endocrinology Unit, Division of Endocrinology, Hospital de Clínicas de Porto Alegre, HCPA, Porto Alegre, Rio Grande do Sul 90035-003, Brazil

(Correspondence should be addressed to S Andrikopoulos; Email: sof@unimelb.edu.au)

Abstract

Infertility, associated with oligo/anovulation, increased ovarian volume, numerous follicular cysts, and metabolic disturbances such as obesity and insulin resistance (IR) are characteristics common to polycystic ovary syndrome (PCOS), the most common endocrine disorder in women of reproductive age. Here, we show that New Zealand obese (NZO) mice display similar metabolic characteristics such as obesity, leptin insensitivity, glucose intolerance, and IR. Importantly, NZO mice are poor breeders; however, the mechanism for this has not been investigated. The aim of this study was to assess the ovarian structure/morphology and sex hormone levels in female NZO and lean C57BL/6J control mice. Twenty-five NZO and twenty female control mice were studied at three different ages (young, adult, and aged). The animals were weighed, an insulin tolerance test was carried out, and blood was collected for measurement of hormone levels. The ovaries were removed for histological

analysis. As expected, NZO mice presented higher body weights ($P=0.001$), increased basal plasma glucose ($P=0.007$), and insulin levels ($P=0.001$) as well as IR, compared with control mice. NZO mice showed an increased ovarian volume, reduced numbers of corpora lutea, and higher total follicle numbers ($P=0.0001$). The number of primordial follicles increased ($P=0.02$) at the young stage, as well as the amount of atretic follicles ($P=0.03$), in NZO compared with control mice. NZO mice also displayed reduced plasma LH and increased estradiol levels. In conclusion, NZO mice show a poor breeding performance due to decreased ovulation, increased number of primordial and atretic follicles, and ovarian size. Given that NZO mice are obese, hyperinsulinemic and insulin resistant, they are suitable for investigating pathophysiological mechanisms linking metabolic alterations with reproductive defects.

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Introduction

Infertility associated with oligo/anovulation, changes in ovarian structure and function, and metabolic disturbances such as obesity and insulin resistance (IR) are characteristics common to polycystic ovary syndrome (PCOS), affecting 5–10% of the female population (Asuncion *et al.* 2000, Lobo & Carmina 2000, Norman 2002, Azziz *et al.* 2004, Norman *et al.* 2004, Ehrmann 2005). In its classic form, it is characterized by oligo/anovulation and hyperandrogenemia. The etiology of PCOS is unclear but evidence suggests that it is a genetically determined ovarian disorder in which excessive androgen production early in life may provide a hormonal insult that leads to PCOS in adulthood (Barnes *et al.* 1994, Crosignani & Nicolosi 2001, Franks *et al.* 2006). The characteristic morphological feature of polycystic ovaries is the accumulation of numerous small antral follicles in

the range of 2–8 mm in diameter (Franks *et al.* 2000), due to an apparent failure to select a dominant follicle, resulting in an abnormal ovarian endocrine environment (Webber *et al.* 2003).

PCOS is also closely associated with metabolic disturbances, most commonly obesity-associated IR (Spritzer *et al.* 2001, Toscani *et al.* 2007) and compensatory hyperinsulinemia (Dunaif 1997, Ehrmann 2005). It is reasonable to suggest that the elevated androgen levels characteristic of PCOS contribute to the increased amount of adipose tissue deposition, particularly in the abdominal region in these women (Barber *et al.* 2006). These metabolic abnormalities have implications both on reproductive function and for long-term health. Weight loss and treatment with insulin-sensitizing drugs (e.g. metformin) can increase the rate of reproduction (Zain & Norman 2008, Leeman & Acharya 2009).

The availability of a suitable animal model would provide a useful means to study the etiology of infertility associated with changes in ovarian structure and function, such as in PCOS. Owing to the heterogeneity of PCOS, it is difficult to create a single-animal model that expresses the main PCOS characteristics. Common models of PCOS include female rats exposed to testosterone (Beloosesky *et al.* 2004) or the aromatase inhibitor letrozole (Kafali *et al.* 2004, Manneras *et al.* 2007) to cause cystic follicles and endocrine features similar to human PCOS. However, these induced models do not develop many of the metabolic disturbances (e.g. increased plasma testosterone, abdominal fat deposition, and IR) that may be important to the disease process. Perhaps, a better model is the prenatally androgenized rhesus monkey that displays hyperandrogenism, anovulation, polyfollicular and enlarged ovaries and metabolic disturbances including increased adiposity and insulin insensitivity (Abbott *et al.* 1998, 2005, 2008). Of course, the usefulness of this primate model is hampered by the specialist vivarium and cost required for such studies.

The New Zealand obese (NZO) mouse is a polygenic model of obesity, IR, and hyperinsulinemia (Veroni *et al.* 1991, Fam *et al.* 2007). One of the earliest described characteristics of the NZO mouse is poor breeding performance such that there was a real possibility that this line may be lost. Of 161 female NZO mice mated at 8 weeks, 94% produced litters and 80% weaned their first litter (Bielschowsky & Bielschowsky 1956). However, only 32% of NZO females had their second litter within 19–25 days of their first, which is strikingly lower than the control mice, with 90% having their second litter within the same interval (Bielschowsky & Bielschowsky 1956). In light of the obesity, IR, and poor breeding performance, we were interested in investigating the NZO mouse further to understand the cause of the poor breeding performance. We hypothesize that the poor breeding performance of the NZO mouse is due to altered ovarian structure and function. The aim of this study was to characterize the reproductive hormone levels and ovarian structure in young, adult and aged female NZO and C57BL/6J control mice.

Materials and Methods

Animals

NZO and C57BL/6J female mice were purchased at 8 weeks of age from the Walter and Eliza Hall Institute of Medical Research (Kew, VIC, Australia). Pups were raised with their biological mother until weaning (21 days of age) and then housed 2–4 per cage under controlled conditions (temperature of 21 °C, humidity of 60%, and kept on an artificial lighting on a 12 h light:12 h darkness photoperiod), and allowed free access to commercial rodent chow (3% fat, 20% protein, and 77% carbohydrate; Ridley Agri Products, Pakenham, VIC, Australia) and tap water. The study was approved by Austin Health Animal Ethics Committee and all the experiments

were in accordance with the guidelines for the care and use of laboratory animals specified by this Committee.

Study procedure

Female NZO and C57BL/6J control mice were studied at 12, 24, and 43 weeks of age, and referred to as young, adult, and aged (Frick 2009). The following procedures were conducted.

Intraperitoneal insulin tolerance test The insulin tolerance test was performed as previously described (Andrikopoulos *et al.* 2000b). Briefly, mice were injected i.p. with 1.0 IU/kg BW of insulin (Actrapid MC; Novo, Bagsvaerd, Denmark) and blood samples (~5 µl) were drawn from the tail vein for the measurement of blood glucose levels on a hand-held Medisense Optium glucometer at 0, 15, 30, 45, and 60 min after the insulin injection.

Hormone assessments Animals were anesthetized with sodium pentobarbital and blood samples were obtained by cardiac puncture after a 6-h fast to assess plasma glucose, insulin, testosterone, estradiol (E₂), and LH concentrations. Plasma samples were stored at –80 °C on the day of extraction and then submitted for RIA. All samples were assayed in duplicate at the Monash Institute of Medical Research (MIMR), Monash University (Clayton, VIC, Australia). Intra-assay coefficient of variation (CV) was 8.2, 8.4, and 12.2% for testosterone, E₂, and LH respectively. As these hormonal measures were done in one assay, there is no inter-assay variation. Plasma glucose levels were measured using a GM7 Analox Glucose Analyzer (Helena Laboratories, Mount Waverley, VIC, Australia), with the linear range being 0–50 mM when using 5 µl of sample and intra-assay CV of 1.4%. Plasma insulin concentrations were determined by a commercially available RIA specific for rat insulin (Millipore, St Charles, MO, USA) with a range of 0.1–10 ng/ml, intra-assay CV of 2.9% and interassay CV of 9.0%.

Histology Ovaries were removed, fixed in Bouin's fixative, dehydrated in ethanol, and embedded in paraffin. Serial sections of 4 µm thickness were stained with hematoxylin-eosin and then evaluated for follicle stages, as described before (Pedersen & Peters 1968), using an Olympus BX50 Phase Pol Darkfield Microscope and MCID Core Software. At least four sections from each ovary were analyzed and the data presented as the mean of the results acquired from right and left ovaries.

The area of the ovary was determined using the virtual microscope and follicles were also measured and counted. The ovarian follicles and corpora lutea (CL) at different stages of development and regression, and the theca and granulosa cell layer were analyzed in detail.

The follicles were counted and classified as healthy or atretic (Brawer *et al.* 1986, Convery *et al.* 1990). Preantral follicles were defined as follicles without any antral cavity and with two or more layers of granulosa cells. Antral follicles were counted when the nucleus of the oocyte was visible

(Lara *et al.* 2000). Follicles presenting normal theca and granulosa cell layers, but devoid of an oocyte, were considered healthy antral follicles due to the limitations of histology that does not always allow its visualization. In this study, the term 'healthy' represented the sum of follicles (preantral, antral, and other healthy follicles) presenting normal granulosa and theca cell layers, not showing deformation or pyknotic cells. Atretic follicles were defined as those follicles exhibiting deformation (shrinkage or collapsing) or lacking the oocyte (Brawer *et al.* 1986, Lara *et al.* 2000) or presenting granulosa cells with pyknotic nuclei (Brawer *et al.* 1986). A cystic follicle was considered to be a large fluid-filled cyst with an attenuated granulosa cell layer and thickened theca interna cell layer (Brawer *et al.* 1986, Lara *et al.* 2000, Manneras *et al.* 2007). Cells facing the cyst were flat and epithelioid, and the cyst fluid invariably contained macrophages (Manneras *et al.* 2007). Type III follicles were defined as large follicular structures (Brawer *et al.* 1986, Desjardins & Brawer 1989) and distinguished from large secondary follicles by virtue of the fact that they did not contain ova. The granulosa membrane was thick and often plicate.

Statistical analysis

Results are expressed as mean ± S.E.M. Comparison between groups was evaluated by the Student's *t*-test, or when multiple comparisons were to be made, a GLM-ANOVA was performed. Homeostasis model assessment for IR (HOMA-IR) was calculated by the following formula: (fasted glucose (mmol/l) × fasted insulin (ng/ml)/22.5) for all animals, as previously reported (Andrikopoulos *et al.* 2008). The area under the curve (AUC) was calculated using the trapezoidal rule. Data were considered to be significant at *P* ≤ 0.05.

Results

Body weight, plasma glucose and insulin levels

Female NZO and C57BL/6J control mice were studied at three ages and the basic metabolic data are shown in Table 1. As expected, both strains of mice increased their body weights

(BW) with aging, with the NZO being heavier at all three time-points compared with control mice. Plasma glucose levels were higher in NZO at the young and adult but not the aged time-point compared with C57BL/6J. Plasma insulin levels were higher at all ages in NZO compared with C57BL/6J, reflective of their insulin-resistant state. Consequently, HOMA-IR, an index of IR, was higher in the NZO compared with C57BL/6J at all ages (Table 1).

Insulin tolerance test

Figure 1A shows plasma glucose levels during an insulin tolerance test and the data are depicted as AUC in Fig. 1B. NZO mice were clearly insulin resistant compared with C57BL/6J mice at all three ages tested, as indicated by greater AUC for glucose following an insulin bolus.

Breeding performance

Table 2 shows breeding performance data of four pairs of NZO and C57BL/6J mice. It is clear that for all parameters tested, including number of litters, number of pups born, number of pups weaned, and therefore survival rate to weaning, the NZO displayed significantly lower numbers compared with the C57BL/6J strain. This is a clear representation of the poor breeding performance of the NZO strain.

Plasma testosterone, LH and E₂ levels

Plasma testosterone levels were not different between NZO and C57BL/6J mice at the young and adult stages (Table 3). However, at the aged stage, there was a significant difference in plasma testosterone with significantly lower levels in NZO mice than in the C57BL/6J control. With age, plasma testosterone levels fell in the NZO female mice, while they increased in the C57BL/6J (Table 3).

Plasma LH levels were significantly reduced in young NZO compared with young C57BL/6J mice (Table 3). Age did not affect plasma LH levels in the C57BL/6J mice, while there was a significant increase in the NZO between young and adult/aged mice (Table 3).

Table 1 Body weight, 6-h fasted plasma glucose, plasma insulin and insulin resistance index (HOMA-IR) in female New Zealand obese and C57BL/6J mice (*n* = 5–12). Values presented as mean ± S.E.M.

Groups	Young		Adult		Aged	
	C57BL/6J (<i>n</i> = 9)	NZO (<i>n</i> = 12)	C57BL/6J (<i>n</i> = 5)	NZO (<i>n</i> = 6)	C57BL/6J (<i>n</i> = 6)	NZO (<i>n</i> = 7)
Age (weeks)	11.5 ± 0.2	11.8 ± 0.6	27.0 ± 0.1	24.0 ± 0.01	45.7 ± 2.3	42.1 ± 1.2
BW (g)	21.3 ± 0.3	35.7 ± 0.8*	24.7 ± 0.7*	46.2 ± 2.2 ^{†,§}	37.7 ± 2.3* [†]	51.6 ± 2.5* ^{†,§}
Glucose (mmol/l)	12.7 ± 0.8	16.0 ± 1.0*	9.6 ± 0.6*	15.0 ± 0.8 [†]	10.9 ± 0.7	12.5 ± 0.7 ^{§,}
Insulin (ng/ml)	0.33 ± 0.04	0.98 ± 0.09*	0.39 ± 0.07	1.16 ± 0.28 [†]	0.48 ± 0.09	2.25 ± 0.98 [†]
HOMA-IR	0.19 ± 0.03	0.68 ± 0.05*	0.16 ± 0.03	0.81 ± 0.22 [†]	0.23 ± 0.04	1.26 ± 0.54 [†]

**P* < 0.05 versus young C57BL/6J; [†]*P* < 0.05 versus adult C57BL/6J; [‡]*P* < 0.05 versus aged C57BL/6J; [§]*P* < 0.05 versus young NZO; ^{||}*P* < 0.05 versus adult NZO.

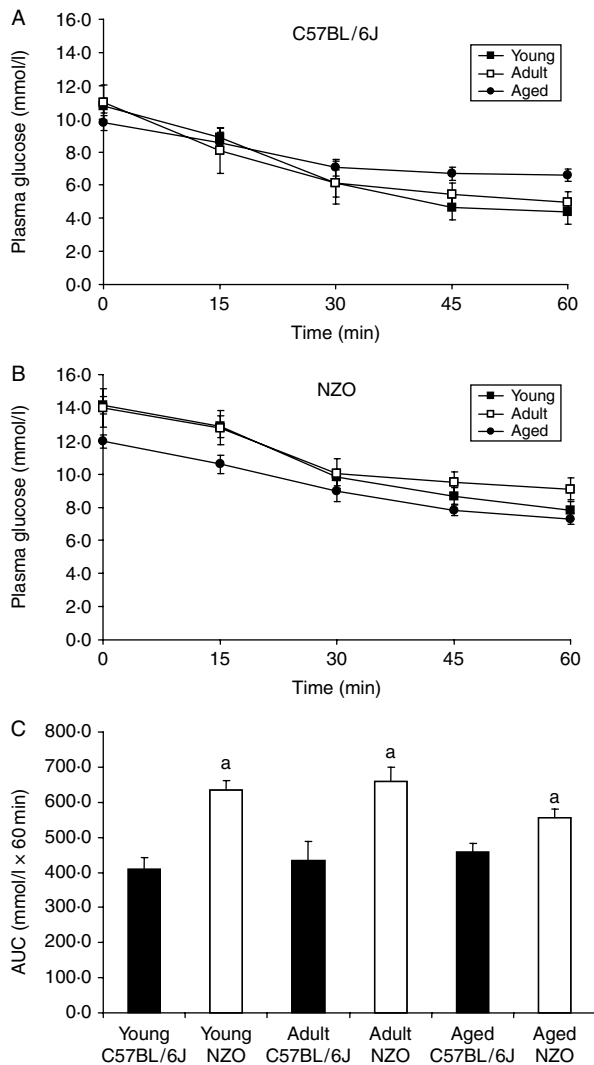


Figure 1 (A) Plasma glucose levels during an insulin tolerance test (1 IU/kg) in female C57BL/6J and NZO mice at the young, adult and aged stages as defined in Materials and Methods. (B) Area under the curve (AUC) of glucose of the insulin tolerance test. Values are presented as mean \pm S.E.M. ($n=6-8$). ^a $P<0.05$ versus C57BL/6J.

In contrast, plasma E_2 levels were higher in young NZO compared with young C57BL/6J mice, while there was no difference at any other time-point (Table 3). There was a significant increase in plasma E_2 levels in young compared with aged NZO mice, while this parameter was actually lower in young compared with aged C57BL/6J mice (Table 3).

Ovarian histology

Histological analysis was performed on ovaries from NZO and C57BL/6J mice at the three different ages, and the images are presented in Fig. 2. Strikingly, the NZO mice had larger ovaries particularly at the young stage with the presence of

antral follicles compared with young C57BL/6J. Healthy follicles were more prominent in C57BL/6J ovaries, with CL evident in both strains in all three ages tested. Quantification of these images was performed, and the data are presented in Figs 3 and 4.

Assessment of ovarian size confirmed the initial histological observations that NZO mice at all three ages had larger ovaries compared with control C57BL/6J mice (Fig. 3A). This difference persisted in the young NZO compared with young C57BL/6J mice even when corrected for BW ($35\,102 \pm 5422$ vs $25\,531 \pm 9360$, $n=8-9$, $P=0.019$). Interestingly, the number of CL was significantly lower in young and adult NZO compared with C57BL/6J mice, indicative of a decreased rate of ovulation (Fig. 3B). There was no difference in CL in aged mice between the two strains. There was a significant positive correlation between fasted plasma insulin levels and ovarian size (Fig. 3C, $r=0.40$, $P=0.013$) when both C57BL/6J and NZO mice were considered together.

The total number of follicles was higher in young and aged NZO compared with C57BL/6J mice (Fig. 4A). The number of primordial (immature) follicles was higher in the young NZO compared with young C57BL/6J mice (Fig. 4B), which may account for the higher total number at this age (Fig. 4A). There was a decrease in primordial follicle number in both strains such that the aged animals had significantly lower numbers compared with young mice (Fig. 4B). When the number of healthy and atretic (unhealthy) follicles was calculated, it was clear that young NZO mice had a higher number of healthy follicles (Fig. 4C) and also a higher number of atretic follicles (Fig. 4D) compared with C57BL/6J mice. The number of healthy follicles declined with age in NZO mice, while there was a significant decrease only in the aged C57BL/6J mice (Fig. 4C). Interestingly, there was no difference in atretic follicle numbers in adult and aged NZO and C57BL/6J mice (Fig. 4D). When the number of healthy and atretic follicles was expressed as a percentage, it is clear that young NZO mice had a higher percentage of atretic follicles compared with C57BL/6J mice (Fig. 4E). There was no difference in the percentage of atretic follicles at the two older ages. Of note, there was a significant increase in the percent of atretic follicles in aged C57BL/6J mice compared with younger mice from the same strain (Fig. 4E).

Table 2 Breeding outcomes of C57BL/6J and New Zealand obese mice. Values presented as means \pm S.E.M.

	C57BL/6J	NZO
Number of litters	21	10
Number of pups born	7.0 ± 0.5	$2.7 \pm 0.4^*$
Number of pups weaned	5.4 ± 0.9	$1.2 \pm 0.7^*$
Survival rate (%)	77.6 ± 11.4	$44.4 \pm 12.2^*$

* $P<0.05$ versus C57BL/6J.

Table 3 Reproductive hormonal levels following a 6-h fast in female New Zealand obese and C57BL/6J mice (n=5–12). Values presented as mean ± S.E.M.

Groups	Young		Adult		Aged	
	C57BL/6J (n=9)	NZO (n=12)	C57BL/6J (n=5)	NZO (n=6)	C57BL/6J (n=6)	NZO (n=7)
Testosterone (pg/ml)	69.8 ± 10.0	61.2 ± 2.4	64.5 ± 7.1	51.4 ± 5.0 [§]	195.5 ± 20.2* [†]	45.9 ± 3.3 ^{†,§}
LH (ng/ml)	0.21 ± 0.04	0.09 ± 0.01*	0.23 ± 0.47	0.27 ± 0.05 [§]	0.48 ± 0.24	0.31 ± 0.07 [§]
Estradiol (pg/ml)	2.7 ± 0.1	5.1 ± 1.4*	3.3 ± 1.3	3.2 ± 1.0 [§]	3.6 ± 1.0*	3.8 ± 1.3 [§]

*P<0.05 versus young C57BL/6J; [†]P<0.05 versus adult C57BL/6J; [‡]P<0.05 versus aged C57BL/6J; [§]P<0.05 versus young NZO.

Discussion

Infertility can be associated with features of the metabolic syndrome, obesity, IR, and polycystic and enlarged ovaries. We assessed the cause of the poor breeding performance of the NZO mouse that is characterized by obesity, hyperinsulinemia, glucose intolerance, and liver and muscle IR (Veroni *et al.* 1991). Furthermore, these mice present with additional defects that are characteristic of the human metabolic syndrome, including dyslipidemia, hypercholesterolemia, and hypertension (Ortlepp *et al.* 2000). In the present study, we confirmed the obese and insulin-resistant state of the NZO mice by measuring BW and performing insulin

tolerance tests. We also confirmed the poor breeding performance of the NZO strain compared with the C57BL/6J. The NZO mouse is an inbred polygenic model of obesity and therefore does not have an appropriate control strain (Andrikopoulos *et al.* 2000a). We have used the C57BL/6J strain for this study as it is generally used as a control strain for many metabolic studies; it is the preferred strain for the generation of genetically modified models (Wong *et al.* 2010) and, according to the JAX website, is considered a good breeder (<http://jaxmice.jax.org/strain/000664.html>). The metabolic features and the well-known poor reproductive rate of the NZO mouse (Bielschowsky & Bielschowsky 1956, Koza *et al.* 2004) provided us with reason

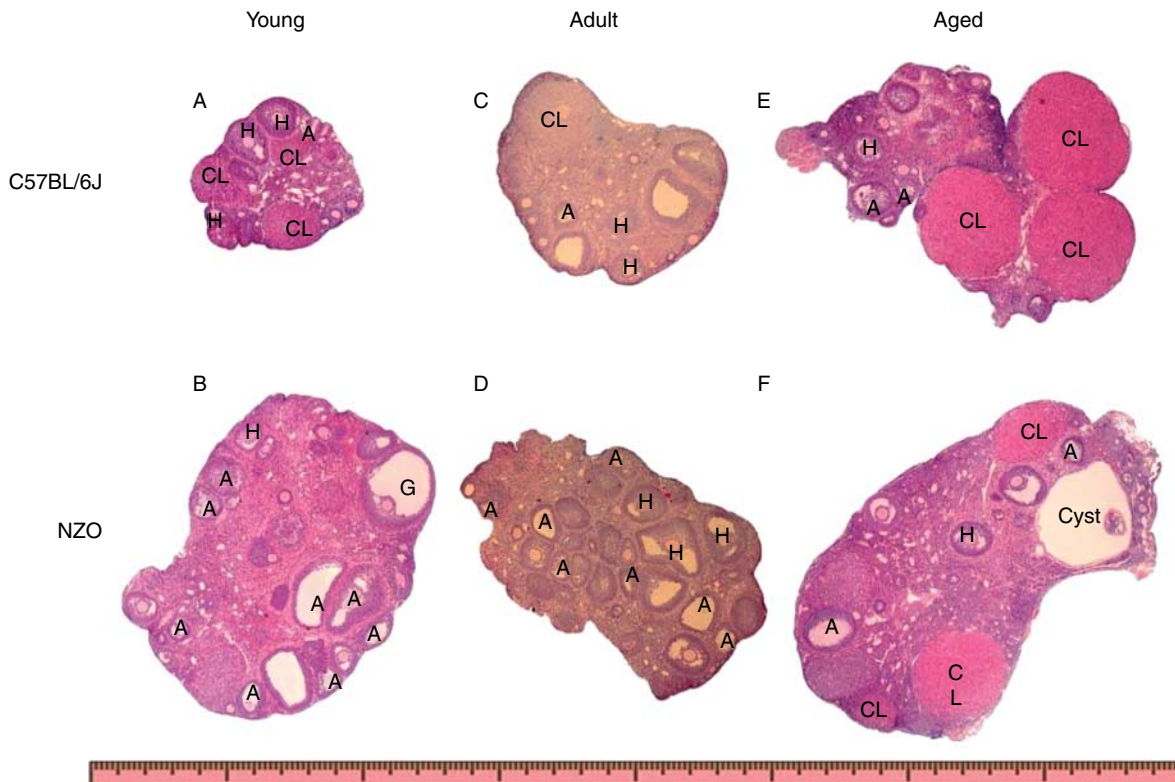


Figure 2 Histological assessment of ovarian structure (125× magnification) in young C57BL/6J and NZO mice (A and B); adult C57BL/6J and NZO mice (C and D); and aged C57BL/6J and NZO mice (E and F). A, atretic follicles; CL, corpora lutea; G, graafian; H, healthy follicles.

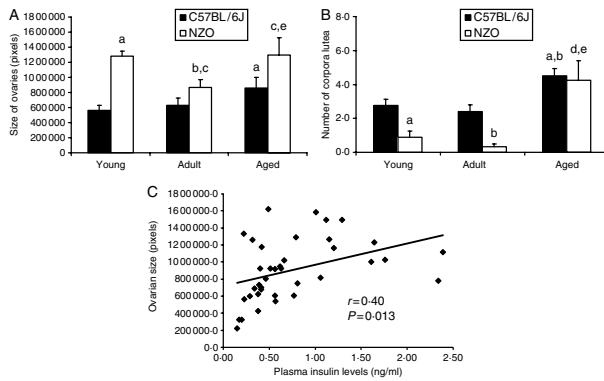


Figure 3 (A) Size of ovaries; (B) number of corpora lutea; (C) correlation between fasted plasma insulin and ovarian size in C57BL/6J and NZO mice. Values are presented as mean \pm S.E.M. ($n=6-8$); $^aP<0.05$; $^bP<0.05$ versus young C57BL/6J; $^cP<0.05$ versus adult C57BL/6J; $^dP<0.05$ versus young NZO; $^eP<0.05$ versus adult NZO.

to study it further. Studying NZO and C57BL/6J mice at different ages allowed us to reach some novel conclusions, specifically, regarding changes in ovarian size, the number of follicles (healthy and atretic) and CL, and reproductive hormonal levels as discussed below.

We showed that the NZO mice had an increased ovarian size, particularly at the young age. This is a well-known morphological characteristic of women with infertility associated with polycystic ovaries and has been shown to correlate with serum insulin and IR markers and with ovarian blood flow (Herter *et al.* 1996, Carmina *et al.* 2005). We also found a significant positive correlation between plasma insulin levels and ovarian size in the mice studied in our experiments (Fig. 3C). While it is certainly plausible, whether insulin is a significant contributor to increased ovarian size has not been formally tested. The number of follicles was also higher in the young NZO mice compared with controls and this is also a feature of polycystic ovaries in humans associated with IR and serum testosterone levels (Hahn *et al.* 2006, Sikka *et al.* 2007). This was most likely contributed to by an increase in the number of primordial follicles in the young NZO mice. An increase in primordial follicular number is a feature of polycystic ovaries and has been recently suggested to be due to a structural change in the ovarian cortex preventing maturation (Woodruff & Shea 2011). Interestingly, while the number of healthy follicles was also increased, the number of atretic follicles was increased to a greater degree such that the ratio of atretic to healthy follicles was higher in the young NZO compared with control mice. The degree of atresia varied in NZO mice, with some follicles showing nuclear pyknosis in only a few periantral cells, whereas others exhibited massive degeneration of the central granulosa cell layers into the antrum, and degenerate cells containing pyknotic nuclei scattered throughout the granulosa membrane. Dying apoptotic cells in atretic follicles can secrete

soluble factors that recruit macrophages (Ravichandran 2003) which appear in the follicular fluid during atresia. The macrophages can then cross and disrupt the basement membrane separating the vascular granulosa membrane from the theca cell layer (Manneras *et al.* 2007), as we observed in our study. Similar changes have been shown in other animal models of induced polycystic ovaries, with ovaries with atretic antral follicles, a thickened theca inner cell layer, a diminished granulosa cell compartment, and few fresh CL (Anderson & Lee 1997, Lara *et al.* 2000, Baravalle *et al.* 2006, Manneras *et al.* 2007).

The number of CL was decreased in young and adult NZO mice compared with controls, suggesting reduced ovulatory rate and explaining the poor breeding performance of this strain (Bielschowsky & Bielschowsky 1956). It has previously been reported that NZO mice present fewer litters than control mice and regular estrous cycle is seen only occasionally in these animals (Bielschowsky & Bielschowsky 1956). Metabolic abnormalities of NZO mice were first reported by Bielschowsky (1953) and the poor breeding was shown 1 year later (Runner & Gates 1954). This is supported by Lara *et al.* 2000, who found a significantly decreased number and size of the CL in rats with steroid-induced PCOS compared with controls. Interestingly, in the aged mice, there was no difference in the number of CL, which likely reflects the prolonged time required for these structures to be sequestered in rodents (Manneras *et al.* 2007).

Of interest is that testosterone levels were not different between NZO and C57BL/6J mice at the young and adult stages. Other models with polycystic ovaries also display

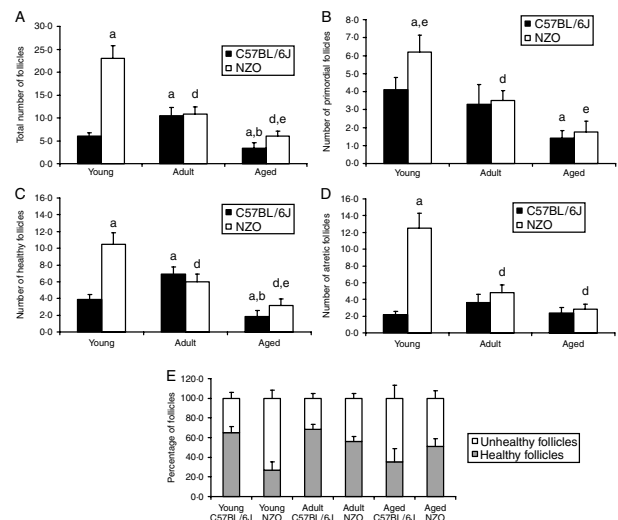


Figure 4 (A) Total number of follicles; (B) number of primordial follicles; (C) number of healthy follicles; (D) number of atretic follicles and (E) percentage of healthy and unhealthy follicles in female C57BL/6J and NZO mice. Values are presented as mean \pm S.E.M. ($n=6-8$). $^aP<0.05$ versus young C57BL/6J; $^bP<0.05$ versus adult C57BL/6J; $^cP<0.05$ versus aged C57BL/6J; $^dP<0.05$ versus young NZO; $^eP<0.05$ versus adult NZO.

normal testosterone levels (Manneras *et al.* 2007) and hyperandrogenemia is not always seen in patients with PCOS (Rotterdam 2004). Furthermore, LH levels were lower in young NZO compared with C57BL/6J mice but no differences were seen at the older ages tested. LH levels actually increased with advancing age in the obese and insulin-resistant NZO mice, in agreement with previous studies in women with PCOS (Piouka *et al.* 2009), including in adolescents with this syndrome (Eagleson *et al.* 2000). However, we are aware that the pulsatile nature of LH release can make LH levels measured at a single time-point difficult to interpret. Similarly, we did observe a significant increase in plasma testosterone levels in aged C57BL/6J mice compared with all other groups. The reason for this is not clear. While a number of studies have assessed testosterone levels at different ages of male C57BL/6J mice (Shapiro *et al.* 1989, Ewart-Toland *et al.* 1999, Krishnamurthy *et al.* 2001, Brouillette *et al.* 2005), such information is not available for female mice. It is important to state that the 2003 Rotterdam consensus criteria state that PCOS can be present and diagnosed without evidence of higher testosterone or LH levels (Rotterdam 2004). In fact, the ovarian dysfunction and PCOS, previously excluded by NIH definition, without hyperandrogenism phenotype are typically characterized with lower LH levels compared with control (Kauffman *et al.* 2007). The characteristics displayed by the NZO mouse (normal androgen levels, increased ovarian size, and number of follicles) represent perhaps 10% of PCOS cases in women. This is evidence of the heterogeneity of the phenotype of PCOS.

Finally, the higher E₂ levels displayed by the young NZO compared with young C57BL/6J mice may be the cause of the decreased LH levels in the obese group, as seen in PCOS (Holte *et al.* 1994, Morales *et al.* 1996, Arroyo *et al.* 1997, Taylor *et al.* 1997, Pagan *et al.* 2006). This increase in E₂ may, in fact, reflect a greater number of follicles in the young NZO mouse, as depicted in Fig. 4C. Interestingly, E₂ levels decreased with advancing age in the NZO mice only as expected (Lu *et al.* 1979, Nelson *et al.* 1995, Frick 2009), since ovarian hormonal levels diminish as menopause approaches in humans, and reproductive senescence in rodents is similar to menopause in several critical respects, including similar alterations in pulsatile LH release and the LH surge, variability of cycle length prior to acyclicity, and ultimate cessation of hormone cycling (LeFevre & McClintock 1988, Nelson *et al.* 1995, Frick 2009).

A limitation of the current study, which is important to note, is that all measurements reported here including circulating hormones and ovarian structure experiments were not matched for the estrous cycle stage. Despite much effort using vaginal swabs, it proved difficult to identify the different stages of the estrous cycle in these mice. It has been reported that the estrous cycle using vaginal swabs is not as discernible in mice as it is in rats (Caligioni 2009). Furthermore, it has been reported that stress can have a significant impact on ovarian hormone levels in rodents,

leading to higher E₂ levels (Garland *et al.* 1987, MacNiven *et al.* 1992, Bowman & Miller 1996, Shors *et al.* 1999). We assessed hormonal levels and harvested ovaries for histological assessment 1 week following the vaginal swabs to ensure that the animals had enough time to recover from the stress of the handling to perform this task. We were able to show that NZO mice presented neither hyperandrogenemia nor primary ovarian insufficiency (evidenced by the absence of low E₂ levels). In addition, ovarian structure data obtained on the same day as blood sample collections confirm functional cycle disturbances in these NZO mice.

In conclusion, this study describes ovarian morphological characteristics in the obese and insulin-resistant NZO mouse that may be well associated with the pathophysiological mechanism involved in the recognized poor reproductive performance of this strain of mice. Therefore, the NZO mouse is a suitable animal model to assess the adverse effects of obesity and IR on ovarian structure and function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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