Impact of nicotinamide mononucleotide on transplanted mouse ovarian tissue

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Abstract

Ovarian tissue cryopreservation and future transplantation is the only strategy to preserve the fertility of young female adolescent and prepubertal patients. The primary challenge to ovarian graft longevity is the substantial loss of primordial follicles during the period of ischaemia post-transplantation. Nicotinamide mononucleotide (NMN), a precursor of the essential metabolite NAD⁺, is known to reduce ischaemic damage. Therefore, the objective of the current study was to assess the impact of short- and long-term NMN administration on follicle number and health following ovarian tissue transplantation. Hemi-ovaries from C57Bl6 mice ($n = 8-12$ /group) were transplanted under the kidney capsule of bilaterally ovariectomised severe combined immunodeficient (SCID) mice. Recipient mice were administered either normal drinking water or water supplemented with NMN (2 g/L) for either 14 or 56 days. At the end of each treatment period, ovarian transplants were collected. There was no effect of NMN on the resumption of oestrous or length of oestrous cycles. Transplantation significantly reduced the total number of follicles with the greatest impact observed at the primordial follicle stage. We report that NMN did not prevent this loss. While NMN did not significantly impact the proportion of apoptotic follicles, NMN normalised PCNA expression at the primordial and intermediate stages but not at later stages. In conclusion, NMN administration did not prevent ovarian follicle loss under the conditions of this study.

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Introduction

Advances in oncology have resulted in dramatically enhanced survival for cancer patients. However, cytotoxic cancer therapies such as ionising radiation and chemotherapy can seriously damage the ovaries of both children and those of reproductive age, leading to premature ovarian failure and consequently a loss of reproductive and endocrine function, a condition with serious long-term hormone-related health outcomes and infertility (Jeruss & Woodruff 2009, Anderson & Wallace 2011).

Fertility preservation is a series of clinical approaches that are targeted to preserve or protect the fertility of cancer patients. Current fertility preservation methods include oocyte and embryo cryopreservation, and ovarian tissue cryopreservation for future transplantation (Jeruss & Woodruff 2009, Anderson & Wallace 2011, De Vos *et al.* 2014, Anderson *et al.* 2015). However, oocyte collection using IVF procedures requires the injection of gonadotrophins

and is, therefore, contraindicated in girls as well as in adult patients with hormone-sensitive cancers and those who do not have time to delay cancer treatment. For these patients, ovarian tissue cryopreservation is the only option currently available (Jeruss & Woodruff 2009, Anderson *et al.* 2015).

Ovarian tissue cryopreservation and transplantation has proven to be an effective clinical procedure with approximately 130 births to date (Donnez & Dolmans 2017) and over 90% return of ovarian function in graft recipients (Donnez & Dolmans 2014, Meiorow *et al.* 2014, Stoop *et al.* 2014). Ovarian tissue cryopreservation and transplantation is a multistep process that leads to a decline in follicle numbers (Amorim *et al.* 2011, Gavish *et al.* 2014, Ayunandari *et al.* 2016, Gavish *et al.* 2018, Roness & Meiorow 2019). The initial removal of ovarian tissue from its physical environment and blood and oxygen supply instigates a deleterious ischaemic cascade of events leading to follicle loss (McCord 1985, Van Eyck *et al.* 2010, Bertoldo *et al.* 2016b, Zhang *et al.* 2017, Roness & Meiorow 2019). This precedes physical

and chemical insults in preparation for and during the cryopreservation and thawing process (Donnez & Dolmans 2010, Lee *et al.* 2016). Additional factors such as graft size, transplantation site and initial ovarian reserve impact the extent of follicle loss (Gavish *et al.* 2014, Roness & Meirow 2019). A diminished ovarian reserve in the graft potentially reduces the long-term endocrine function and reproductive potential of the graft, requiring additional transplantations of tissue from a valuable and limited supply.

Of the multiple steps involved in a cryopreservation and transplantation procedure, transplantation of ovarian tissue causes the most significant insult to follicle numbers (Amorim *et al.* 2011, Gavish *et al.* 2018, Roness & Meirow 2019). The period immediately post-transplantation is characterised by ischaemia and an acute loss of both growing and primordial follicles with only 5–30% of primordial follicles surviving the grafting process (Baird *et al.* 1999, Liu *et al.* 2002, Dolmans *et al.* 2007, Amorim *et al.* 2011, Gavish *et al.* 2018). Furthermore, ischaemia promotes fibrosis and is a threat to graft survival (Dath *et al.* 2010). In order to reinstate blood supply, neoangiogenesis takes approximately 3–5 days (Martinez-Madrid *et al.* 2009, Van Eyck *et al.* 2009). During this time, a significant proportion of primordial follicles activate, leading to an increase in the proportion of growing follicles and proliferation of granulosa cells (Amorim *et al.* 2011, Gavish *et al.* 2014, 2018). Follicle survival depends on rescuing the tissue from ischaemia and hypoxia with neovascularisation. Therefore, strategies to enhance the quality of transplanted ovarian tissue are required to improve fertility preservation outcomes for cancer patients.

Nicotinamide adenine dinucleotide (NAD⁺) is an essential cellular cofactor involved in several critical cell functions including redox reactions, ATP generation, DNA repair and genome stability (Canto *et al.* 2015, Bonkowski & Sinclair 2016). In mammals, NAD⁺ is synthesised by four biosynthetic pathways, including *de novo* synthesis from tryptophan, the Preiss–Handler pathway from nicotinic acid, the nicotinamide riboside pathway using nicotinamide riboside, or conversion from nicotinamide in the salvage pathway (Canto *et al.* 2015, Fang *et al.* 2017). Synthesis of NAD⁺ via the salvage pathway is the cell's primary source of NAD⁺, involving the conversion of nicotinamide to nicotinamide mononucleotide (NMN) via the action of nicotinamide phosphoribosyltransferase (NAMPT), which is then, in turn, catalysed to NAD⁺ by nicotinamide mononucleotide adenylyltransferase (NMNAT) (Mori *et al.* 2014, Fang *et al.* 2017). NMN, the rate-limiting intermediate of the salvage pathway, has gained significant attention as a NAD-boosting molecule with therapeutic potential (Rajman *et al.* 2018).

It is well established that levels of NAD⁺ decline during ischaemic injury (Yamamoto *et al.* 2014,

Park *et al.* 2016). To maintain NAD⁺ levels following ischaemic injury, NAD⁺ consuming processes can be inhibited, or NAD⁺ generating processes enhanced via the salvage pathway using the NAD⁺ precursor NMN. Administration of NMN protected heart tissue (Yamamoto *et al.* 2014), muscle (Das *et al.* 2018) and prevented brain damage (Park *et al.* 2016) from ischaemic insult. Furthermore, we and others recently demonstrated that NAD⁺ precursor administration to mice reversed age-induced reductions in oocyte quality and fertility (Bertoldo *et al.* 2020, Miao *et al.* 2020, Yang *et al.* 2020). Taken together, these data suggest that NMN administration could enhance ovarian graft outcomes. Since NAMPT is a rate-limiting enzyme in the salvage pathway that results in depressed levels of NMN, we hypothesised that NMN administration could preserve follicle numbers in grafts of ovarian tissue. The aim of the present study is to assess the efficacy of NMN as a clinically relevant strategy to preserve follicle populations in ovarian tissue transplants.

Materials and methods

Mice

All animal experiments were approved by the University of New South Wales (UNSW) Animal Ethics Committee. UNSW AEC operates under animal ethics guidelines from the National Health and Medical Research Council (NHMRC) of Australia. Female C57Bl/6 mice were purchased from Australian Bio-Resources (ABR; Moss Vale, NSW, Australia). Female severe combined immunodeficient (SCID) mice were purchased from Animal Resources Centre (ARC; Perth, WA, Australia). Animals were maintained in ventilated cages at 22°C and 80% humidity at a density of up to 5 per cage, with access to food and water *ad libitum*. All water in this animal house was acidified to pH 3 with HCl to decrease microbial growth. NMN (GeneHarbor Biotechnology, Hong Kong, China) was added to drinking water at 2 g/L as previously described (Bertoldo *et al.* 2020). The UNSW Lowy Animal Facility maintained a 12 h light: 12 h darkness cycle with lights on at 07:00 h and off at 19:00 h. Twenty-four hours before surgery, recipient SCID females were randomly allocated to two groups and provided either standard water or water supplemented with NMN. Two experiments were conducted with differing durations of NMN treatment; for 14 days (Experiment 1); and for 56 days (Experiment 2). Schematic descriptions of the experiments are shown in Fig. 1. Given revascularisation of ovarian tissue occurs over a period of 3–5 days (Martinez-Madrid *et al.* 2009, Van Eyck *et al.* 2009), and a previous study had demonstrated that follicle development in transplanted tissue could be modulated 7 days after transplantation (Gao *et al.* 2013), the initial timepoint of 14 days was selected to allow grafted tissue to undergo two oestrous cycles before assessing follicle numbers. The second timepoint of 56 days was selected to assess long-term effects on follicle

Figure 1 Schematic of Experiment 1 (A) examining the effect of 14 days of NMN treatment, and Experiment 2 (B) with 56 days of NMN treatment. In both experiments, hemi-ovaries from adult (6 weeks old) female C57Bl6 mice were transplanted without prior cryopreservation under the kidney capsule of recipient adult (10-week-old) SCID mice, or were immediately fixed (day 0 non-transplanted control). On the day before surgery (day -1) SCID mice were randomly allocated to either normal water (control) or NMN (2 g/L) in drinking water, and treatments were maintained for 14 days (Experiment 1; A) or 56 days (Experiment 2; B). At the conclusion of surgery (day 0) and the day after surgery (day 1) recipient mice received an i.p. injection of either saline or NMN (200 mg/kg). Ovaries were collected after 14 (A) or 56 (B) days.

development and to our knowledge, there were no previous reports reliably assessing transplanted mouse tissue past 3 weeks post-transplantation.

Ovarian tissue collection

Donor C57Bl/6 mice (4–6 weeks of age) were sacrificed by cervical dislocation. Ovaries were collected and placed into HEPES-buffered α MEM (GIBCO Life Technologies) handling medium supplemented with 3 mg/mL BSA (Sigma Aldrich). Under a dissecting microscope each ovary was trimmed of excess tissue and cut in half using a razor blade. Of the four hemi-ovaries from each donor animal, one was randomly allocated to fixation in 4% PFA overnight at 4°C representing the day 0 non-transplanted control, another two randomly allocated to either the control group or NMN group (see next section), and the fourth hemi-ovary was discarded. Eleven donors and 22 recipients were used in Experiment 1, and 8 donors and 16 recipients in Experiment 2.

Ovariectomy and transplantation into SCID mice

All surgeries were performed on warming mats set at 37°C. To perform bilateral ovariectomies, recipient SCID mice (8–10 weeks old) were anaesthetised with 2.5–3% isoflurane (Henry Schein, Mascot, Australia) administered via a nose cone. A

single injection of carprofen (5 mg/kg; Carprive, Norbrook) in sterile saline was administered as analgesic. An abdominal lateral incision at the level of the kidney was made, the wall of the abdomen and peritoneum opened, and the ovary and uterine horn exteriorised. The ovarian vasculature was tied off and restricted with an 8-0 silk suture (Ethicon, Puerto Rico, USA), the ovary removed, and the reproductive tract returned to its normal anatomical position. The peritoneum was sutured closed with 6-gauge suture and abdominal wall closed with either a 4-0 silk suture or 7 mm Michel clips (CellPoint Scientific, Maryland, USA). On the contralateral side, the same process was repeated, however, before closing the peritoneum and abdominal wall, the kidney was exposed, and a small tear was made in the kidney capsule. A piece of ovarian tissue was randomly selected and inserted under the kidney capsule as previously described (Gook *et al.* 2001). The peritoneum and abdominal wall were then closed and the mouse administered either 100 μ L of saline (control group) or NMN (200 mg/kg in 100 μ L of saline) (Ho *et al.* 2017, Marinova *et al.* 2020) and placed in a warmed cage to recover. All procedures were performed under aseptic conditions. Twenty-four hours after surgery, mice were administered a second injection of saline or NMN. The intraperitoneal injections were administered in the first 24 h to provide sufficient NMN directly into the peritoneum as an alternative route of NMN administration for ovarian tissue protection as previously described (Ho *et al.* 2017, Marinova *et al.* 2020).

Assessment of the oestrous cycle

In Experiment 2, oestrous cycle stage was determined from vaginal epithelial cell smears taken daily for 12 consecutive days, commencing on day 5 post-surgery (Fig. 1). Smears were collected using 15 μ L of 0.9% sterile saline and transferred to glass slides to air dry. Dry smears were stained with 0.5% toluidine blue before being examined under a light microscope. Oestrous cycle stage was determined based on the presence or absence of leukocytes, cornified epithelial cells, and nucleated epithelial cells. Proestrus was characterised by the presence of mostly nucleated and some cornified epithelial cells; oestrus was identified by the presence of primarily cornified epithelial cells; at metoestrus both cornified epithelial cells and leukocytes were present; and at dioestrus predominantly leukocytes were present.

Graft recovery and histological assessment

At the end of the treatment period (Experiment 1, 14 days; Experiment 2, 56 days), SCID mice were anaesthetised using 3% isoflurane via a nose cone. Mice were then killed by cervical dislocation and the entire kidney with the graft was recovered and fixed in 4% PFA overnight at 4°C. The next day the majority of the renal tissue was removed using a scalpel being cautious not to disturb the graft, with the graft then stored in 70% ethanol before histological processing. The entire graft with remaining renal tissue was then embedded in paraffin and cut into serial sections, 5 μ m in thickness. Every 10th serial section was stained with haematoxylin and eosin (H&E) and observed with light microscopy to perform

histomorphologic assessments. The remaining sections were kept for immunohistochemical staining. Ovarian follicles were classified as primordial, intermediate, primary, secondary and antral, following standard criteria (Myers *et al.* 2004, Bertoldo *et al.* 2016a). Primordial follicles were characterised by one oocyte surrounded by a single layer of flattened pre-granulosa cells. Intermediate follicles consisted of an oocyte surrounded by a single layer of flattened and cuboidal granulosa cells. In primary follicles, oocytes were surrounded by at least one complete layer of cuboidal granulosa cells. Secondary follicles contained at least two complete layers of cuboidal granulosa cells. Antral follicles were characterised by the development of an antral cavity. At least 134 and 69 sections were assessed for Experiments 1 and 2, respectively.

To evaluate the effect of transplantation and NMN on oocyte and follicle growth, oocyte and follicle diameters were calculated from the mean of two perpendicular measurements using the computer-based image analysis program ImageJ Software (NIH, USA). The boundary of the follicle was defined as the basement membrane, clearly visible as a demarcation between the granulosa cells and the surrounding theca/stroma. Only follicles completely free of any sign of atresia such as pyknotic cells were analysed for morphometric assessments. All histological assessments were performed blinded by two independent observers.

Immunohistochemistry and image analysis

Tissue was deparaffinised in xylene, rehydrated and endogenous peroxidases blocked with 1.2% hydrogen peroxide. Sections were then blocked with 1% fetal bovine serum (FBS; GIBCO) and washed in PBS. Those sections destined for analysis of caspase-3 expression were subjected to heat-induced antigen retrieval using sodium citrate (Vector Labs H3300). Sections were incubated overnight at 4°C in a humidified chamber with either rabbit anti-caspase-3 (diluted 1:400 in PBS + 1% FBS; Abcam, ab13847) or rabbit anti-PCNA (diluted 1:400 in PBS + 1% FBS; Sigma, SAB2108448). Sections were washed three times in PBS and then incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, cat# 111-035-144) diluted 1:800 in PBS with 0.1% BSA at room temperature for 4 h in a humidified chamber. Immunostaining was developed by using DAB substrate (Vector Labs) as a chromogen for 70 s. Doxorubicin (Cayman Chemical) treated mouse ovarian tissue and paraffin-embedded mouse intestinal tissue were used as positive controls for caspase-3 and PCNA, respectively. Rabbit isotope control IgG (Abcam, ab172730) in place of the primary antibody served as a negative control. All samples were processed at the same time to prevent inter-replicate variation in staining. Images were captured with a computer-based program (Olympus DP Controller, Olympus Optical Japan) and analysed using ImageJ Software. The proportion of caspase-3 immunopositive granulosa cell area was calculated as a percentage of total antral follicle area and evaluated through colour segmentation analysis, which identifies objects by locating all objects of a specific colour

(brown stain for DAB chromagen), as described previously (Ortega *et al.* 2009, Bertoldo *et al.* 2019). To assess only granulosa cell caspase-3 protein expression, the oocyte and antral cavity were digitally removed from the images before analysis. PCNA expression was calculated as a proportion of label-positive granulosa cells from the total granulosa cell number within each follicle (Salveti *et al.* 2012). Sections were analysed with the observer blinded to treatment.

Follicle health

In Experiment 2, the extended time period resulted in insufficient numbers of follicles to adequately assess PCNA and cleaved caspase-3. Therefore, as an alternative to evaluate follicle health, we assessed the proportion of pyknotic granulosa cells which requires far fewer follicles than would otherwise be required for immunohistochemistry. Unhealthy primary, secondary and antral follicles were classified according to strict morphometric criteria if they contained a degenerate oocyte, disorganised granulosa cells layers and/or more than 10% of the granulosa cells being pyknotic in appearance, as previously described (Walters *et al.* 2007). The proportion of unhealthy follicles in each graft was calculated as the percentage of all follicles at that developmental stage. All health assessments were performed blinded.

Statistical analysis

Differences in the total number of follicles were analysed by one-way ANOVA followed by a Tukey's multiple comparison test. Differences in the proportion of growing vs non-growing and proportion of follicles at each stage were analysed using a Kruskal–Wallis test following arcsine transformation of percentage data. Oocyte and follicle diameters and the oocyte:follicle ratio were analysed using either one-way ANOVA followed by a Tukey's *post hoc* test or a Kruskal–Wallis test. PCNA and cleaved caspase-3 expression was analysed using a Kruskal–Wallis test followed by a Dunn's *post hoc* test. Oestrous cycling data were analysed using a Mann–Whitney test, while differences in the proportion of unhealthy follicles were analysed by χ^2 . Statistical differences $P < 0.05$ were considered significant. Data are shown in figures as mean $\pm$ 95% CI.

Results

Experiment 1

Follicle distributions

At least 134 sections in each group were assessed for follicle distribution data. Fourteen days after transplantation overall ovarian graft morphology was grossly similar in both transplanted groups. Grafted tissue in the control and NMN groups contained significantly fewer follicles compared to fresh tissue ($P < 0.001$), with no significant differences between transplant groups (Fig. 2A and Supplementary Fig. 1A, see section on supplementary materials given at the end of this article).

Figure 2 Follicle numbers and the proportion of growing follicles in day 0 non-transplanted control ovaries and in ovarian grafts in recipients administered either normal water or water supplemented with NMN (2 g/L). On day 14 (D14) ovarian transplants were collected and (A) follicle number per section, (B) the proportion of non-growing and growing follicles, and (C) the proportion of follicles at each stage of follicle development were determined and compared to day 0 non-transplanted tissue from the same animals. Data are presented as mean $\pm$ 95% CI (A, C). Number of hemi-ovaries assessed=11 per treatment. *** $P < 0.0001$; ** $P < 0.001$; * $P < 0.05$.

The proportion of non-growing and growing follicles in non- and transplanted tissue are presented in Fig. 2B. All grafts exhibited significant increases in growing follicles compared to fresh tissue ($P < 0.05$). When follicles were classified according to developmental stage, it was observed that a significant reduction in the proportion of primordial follicles in both transplanted groups was associated with a concomitant increase in the proportion of primary follicles (Fig. 2C; $P < 0.05$), inferring that primordial follicle activation had taken place as a result of transplantation (Fig. 2C). At the antral stage, there was a small but significant reduction in the number of antral follicles in control transplanted tissue compared to non-transplanted tissue ($P < 0.05$; Fig. 2C).

Follicle morphometrics

Oocyte and follicle diameter and the oocyte to follicle ratio were assessed. As presented in Fig. 3A, compared to day 0 tissue, there was a significant reduction in oocyte size in secondary follicles from both control and NMN grafted tissue ($P < 0.0001$), which was not observed in either primary or antral follicle stages. This was associated with a significant reduction in secondary follicle size (Fig. 3B). However, when follicles in grafted tissue reached the antral stage, they were significantly larger compared to antral follicles from day 0 fresh tissue ($P < 0.05$; Fig. 3B). Consequently, the oocyte to follicle ratio of antral follicles was smaller in grafted tissue compared to fresh tissue (Fig. 3C). There were no effects of NMN treatment on oocyte or follicle size at any stage of follicle development, compared to control grafts (Fig. 3).

Granulosa cell apoptosis and proliferation

PCNA staining in an antral follicle from the NMN treatment is shown in Fig. 4A. Analysis of 548 follicles

showed that transplantation led to a notable increase in PCNA expression at all stages of follicle development, compared to the day 0 non-transplanted control ($P < 0.05$; Fig. 4B). While PCNA expression in grafts from NMN-treated animals, compared to control grafts, appeared to be more similar to that of fresh non-transplanted tissue at the primordial and intermediate stages ($P > 0.05$), primary, secondary and antral follicle PCNA expression in NMN-treated transplanted tissue was significantly greater ($P < 0.05$) than that of fresh tissue (Fig. 4B). A representative example of cleaved caspase-3 immunostaining in an antral follicle from the NMN treatment, is shown in Fig. 4C. Overall expression of cleaved caspase-3 (apoptosis) was broadly similar in both transplanted groups. Following analysis of 608 follicles, cleaved caspase-3 expression levels were similar between fresh non-transplanted and transplanted tissues treated with or without NMN, at all follicle stages (Fig. 4D).

Experiment 2

Oestrous cycling

Examples of oestrous cyclicity in females from each group are represented in Fig. 5A. Assessment of oestrous cycle resumption commenced 5 days after transplantation surgery and continued for 12 consecutive days. The recovery rate for oestrous cyclicity was 100% in both groups. Compared to the control group, there was no significant effect of NMN treatment on the period of time animals spent at each stage of the oestrous cycle (Fig. 5B), nor on cycle length (Fig. 5C).

Follicle distributions

At least 69 sections in each group were assessed for follicle distribution data. Fifty-six days after

Figure 3 Oocyte and follicle diameter and oocyte:follicle ratio from primary, secondary and antral follicles in day 0 non-transplanted ovarian tissue compared to ovarian grafts in recipients administered either normal water or water supplemented with NMN (2 g/L). Day 0 or day 14 ovarian transplants were collected and (A) oocyte diameter, (B) follicle diameter and, (C) oocyte:follicle ratio at each stage were determined. Measurements were collected from 11 animals in each group. Data are presented as mean $\pm$ 95% CI. *** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

transplantation, overall ovarian graft morphology was broadly similar in control and NMN-treated recipients. In general, the pattern of follicle loss as a result of transplantation and subsequent follicle development was similar to that of Experiment 1, but was more accentuated due to the extended period the grafts remained *in vivo*. Grafted tissue in the control and NMN groups contained significantly fewer follicles compared to day 0 fresh non-transplanted tissue ($P < 0.001$), and there was no significant difference between the transplant groups (Fig. 5D and Supplementary Fig. 1B). The proportion of non-growing and growing follicles in non-transplanted and transplanted tissues are presented in Fig. 5E. All grafts exhibited a significant increase in the proportion of growing follicles compared to the fresh non-transplanted tissue ($P < 0.001$; Fig. 5E). Follicle developmental stage classification showed a substantial and significant ($P < 0.0001$) reduction in the proportion of primordial follicles in both transplanted groups, compared to the non-transplanted day 0 control, which was associated with a concomitant increase ($P < 0.001$) in the proportion of secondary and antral follicles, inferring transplantation led to the activation of primordial follicles and that follicle development had proceeded throughout the 56-day experiment (Fig. 5F).

Follicle morphometrics and health

As presented in Fig. 6A, oocyte diameter in primary and secondary follicles were similar between all groups. However, at the antral stage, NMN treatment of transplanted animals significantly ($P < 0.001$) reduced oocyte diameter compared to those in non-transplanted tissue ($P < 0.001$). There was no effect of transplantation or treatment of grafted animals with NMN on follicle diameter ($P > 0.05$; Fig. 6B). The oocyte to follicle ratio of secondary and antral follicles was lower ($P < 0.05$) in grafted tissue, irrespective of NMN treatment, compared to fresh tissue (Fig. 6C).

To assess follicle health after 56 days of transplantation, the number of pyknotic granulosa cells were counted in primary, secondary and antral follicles, and compared to day 0 fresh non-transplanted tissue. There was no significant effect of transplantation or NMN treatment on the proportion of unhealthy follicles (Fig. 6D).

Discussion

Strategies to prevent follicle loss in transplanted ovarian tissue are required to enhance outcomes for this fertility preservation approach. In the present study, we investigated the potential for NMN administration to enhance ovarian tissue graft outcomes by preventing follicle loss in a mouse model. However, we report that NMN did not prevent follicle loss in transplanted tissue.

Figure 4 Granulosa cell proliferation and apoptosis in primordial, intermediate, primary, secondary and antral follicles on day 0 non-transplanted ovarian tissue, compared to ovarian grafts in recipients administered either normal water or water supplemented with NMN (2 g/L) for 14 days. (A) Representative images of PCNA expression, (B) the proportion of PCNA positive cell cells, (C) representative images of cleaved caspase-3 expression and (D) cleaved caspase-3 immunopositive area was calculated. Arrow heads identify representative positive cells for both PCNA and cleaved caspase-3. Data are presented as mean $\pm$ 95% CI. *** P < 0.0001; ** P < 0.001; * P < 0.05; a, 0.06. Scale bar = 100 μ m.

Declining cellular NAD⁺ levels are a feature of ischaemia (Wang *et al.* 2011, Yamamoto *et al.* 2014, Park *et al.* 2016, Das *et al.* 2018). Previous animal studies have demonstrated that NMN treatment prevents ischaemic damage in the brain, heart and muscle, which is associated with reduced cell death and improved tissue function (Wang *et al.* 2011, Yamamoto *et al.* 2014, Park *et al.* 2016, Wei *et al.* 2017, Das *et al.* 2018). While the underlying mechanisms are unclear, some evidence suggests that the NMN-NAD-SIRT1 and NMN-NAD-PARP pathways play a key role in protecting against neural and cardiac ischemia (Wang *et al.* 2011, Yamamoto *et al.* 2014, Liang *et al.* 2015). Sirtuins, a family of protein deacylases, regulate many fundamental processes within cells including cell metabolism, DNA repair, maintenance of genome integrity, cellular senescence and inflammation (Rajman *et al.* 2018, Tatone *et al.* 2018), and are implicated in fertility (Di Emidio *et al.* 2014, Bertoldo *et al.* 2016b, 2020, Iljas *et al.* 2020). A second family of NAD⁺ consuming enzymes are the poly-ADP-ribose polymerases (PARPs). The most understood member of the family, PARP1, plays a key role in the repair of single-strand DNA breaks resulting

from oxidative stress. Paradoxically, PARPs also increase ischaemic damage (Endres *et al.* 1997, Cozzi *et al.* 2006), and when PARP1 function is disrupted, mice are resistant to cerebral ischaemia (Eliasson *et al.* 1997). Evidence suggests that PARP1 activity increases with decreasing NAD⁺ concentration in mouse tissue, which was negated by NMN administration, suggesting that NAD⁺ is consumed by PARP1 to repair tissue. However, NMN inhibited PARP1-dependent NAD⁺ catabolism (Park *et al.* 2016). Therefore, the inhibition of poly-ADP-ribose formation by NMN suggests that the protective mechanism is not due to an increase in NAD⁺ availability via the salvage pathway, but rather inhibition of PARP1 activity following ischaemic insult (Park *et al.* 2016). We did not directly assess levels of ischaemia in grafted tissue. However, given we found that NMN did not impact follicle numbers or apoptosis, we surmise that NMN administered systemically to recipients did not protect against ischaemic damage in grafted tissue. This contrasts with the previously described beneficial effects of NMN against ischaemic damage in other organs.

In the present study, we commenced NMN treatment (in drinking water) the day before surgery. At the

Figure 5 Effect of transplant and NMN on oestrous cycle and follicle development. Starting 5 days after transplantation surgery vaginal smears were taken for 12 days. (A) Representative oestrous cycles from control and NMN-treated recipient females, (B) the number of days at each cycle stage, and (C) average oestrous cycle length were assessed. On day 56 (D56) ovarian transplants were collected and (D) follicle number per section, (E) the proportion of non-growing and growing follicles, and (F) the proportion of follicles at each stage of follicle development were determined and compared to day 0 non-transplanted tissue. Data are presented as mean $\pm$ 95% CI (A, B, C, E). Number of hemi-ovaries assessed = 8 per treatment. **** $P < 0.0001$; *** $P < 0.0001$; ** $P < 0.001$; * $P < 0.05$.

completion of surgery and the day after surgery, animals were administered an i.p. injection of NMN (200 mg/kg) as this regime has previously been shown to protect ovarian tissue against chemotherapy (Ho *et al.* 2017, Marinova *et al.* 2020). We did not confirm if NMN and NAD⁺ levels were raised in grafted tissue, however, Yamamoto *et al.* (2014) demonstrated that i.p. injection of NMN (500 mg/kg) successfully increased NAD⁺ content in the heart within 30 min, and that treatment with NMN 30 min before cardiac ischaemia avoided ischaemia-induced reductions in NAD⁺ content in the heart, suggesting that i.p. administration of NMN enhances the amount of NAD⁺ of the heart during ischaemia within a relatively short period (Yamamoto *et al.* 2014). While the model by Yamamoto *et al.* (2014) is different to the present study, whereby cardiac blood supply was cut-off then reinstated, whereas the present experimental model required neovascularisation between ovarian and renal tissue, it is possible that injecting recipient mice before surgery and with a larger dose may have yielded a different outcome and prevented follicle loss. Irrespective of the differences in treatment regimes, NMN treatment in the current study did not prevent follicle loss as a result of transplantation.

Recently, we were the first to demonstrate, in the context of reproductive ageing, a beneficial effect of NMN therapy on several readouts of fertility, including increased ovulation rate and litter size, suggesting that NMN modulates follicle development (Bertoldo *et al.* 2020). These initial findings were independently validated (Miao *et al.* 2020, Yang *et al.* 2020), and together drive significant interest in the broader potential application of NAD⁺ boosting compounds in reproductive medicine, and in the case of the present study, investigating the potential benefit of increasing cellular NAD⁺ to mitigate against follicle loss in ovarian tissue grafts. However, recently, Stringer *et al.* (2020) reported that NMN failed to protect against depletion of the ovarian reserve following cancer treatment. In that study, which aimed to determine whether supplementation of NMN could preserve the ovarian reserve in intact animals following γ -irradiation or cyclophosphamide treatment, NMN treatment was provided 7 days before cancer treatment and then stopped the day after cancer treatment, precisely when we would predict the greatest support for NAD⁺ synthesis is needed to protect the ovary. Therefore, it is not unexpected that NMN did not provide protection to the ovarian reserve in that study.

Figure 6 Oocyte and follicle diameter and oocyte:follicle ratio from primary, secondary and antral follicles in day 0 non-transplanted ovarian tissue compared to ovarian grafts in recipients administered either normal water or water supplemented with NMN (2 g/L). Grafts were recovered 56 days after surgery and the (A) oocyte diameter, (B) follicle diameter, (C) oocyte:follicle ratio and, (D) proportion of unhealthy follicles at each stage of follicle development were determined. Data are presented as mean $\pm$ 95% CI (A, B, C). ** $P < 0.001$; * $P < 0.05$.

This contrasts with the present study where we supplied NMN before and after surgery. However, it is possible that we did not administer NMN long enough prior to surgery as we previously demonstrated that NMN supplied in drinking water 3 days before doxorubicin and cisplatin treatment and continued for a further 8 weeks, protected against depletion of the ovarian reserve (Ho *et al.* 2017, Marinova *et al.* 2020). Taken together, this implies that for NMN to provide a protective effect, a sufficient period of time before and after insult (grafting or chemotherapy) is required.

Indeed, the age of the donors may have contributed to the results of the present study since tissue from young animals is perhaps refractory to ischemia. However, follicle loss following ovarian tissue transplantation occurs even when transplanted tissue originates from young (13–26 years) patients (Gavish *et al.* 2018) and young mice at similar ages to the donors in the present study (Mahmoodi *et al.* 2015). Ischemic damage also occurs in ovaries of prepubertal rodents (Usta *et al.* 2008, Tuncer *et al.* 2016), suggesting that ischemia occurs at all ages.

Given we observed minimal effects of NMN on follicle number and health, a potential concern is that our graft technique is not adequate. However, this is unlikely for two primary reasons. First, 100% of the graft recipients, which had none of their own ovarian tissue as they were ovariectomised, exhibited resumption of oestrous cyclicity and with cycles of the correct length. Secondly, 14 days after transplantation, the reduction in the proportion of primordial follicles was associated with a concomitant increase in primary follicles only, and after 56 days, a concomitant increase in secondary and antral follicles was observed. Taken together, these observations demonstrate that the grafts were able to support follicle development through to the antral stage with an associated resumption of systemic endocrine function, and therefore the grafts were functional and could be expected to be responsive to interventions. Alternatively, high endogenous levels of renal NAD⁺ and NMN may also have impacted graft outcomes (Mori *et al.* 2014), thereby masking any beneficial effect of NMN administration.

In conclusion, the current study has shown that the NMN treatment regimes described do not prevent follicle loss in ovarian tissue grafts from adult C57Bl/6 mice. We also demonstrated that NMN does not appear to greatly impact follicle health or viability. The observation that at 14 days post-surgery, secondary follicles in transplanted tissue were smaller than those in fresh tissue but at the antral stage are larger than fresh tissue, may suggest a level of asynchrony in granulosa cell proliferation (Smitz & Cortvrindt 2002) and subsequent compensatory development. Given ovarian tissue transplantation and the preceding period of ischaemia is a significant insult to tissue viability,

the treatment regimes in the present study may have to be adapted to better protect tissue. Nonetheless, further research is required to determine if modifying the dose or mode of administration of NAD⁺ boosting compounds will protect the ovarian reserve from insult during transplantation.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0539>.

Declaration of interest

The salary of MB in the labs of R B G and L E W was partly supported by sponsored research from Jumpstart Fertility Pty Ltd. L E W is a founder, shareholder and director of Jumpstart Fertility. He is also a consultant to the Life Biosciences group, and is a scientific advisor and shareholder to Metro Biotech, a member of the EdenRoc group, and to Intravital Pty Ltd. R B G is a consultant to City Fertility Centre Global Pty Ltd. Robert Gilchrist is on the editorial board of Reproduction. Robert Gilchrist was not involved in the review or editorial process for this paper, on which he is listed as an author.

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Author contribution statement

M J B, R B G and K A W conceived and planned the study. M J B, V R P, D A G, M C E, K W, C J J L and M B M performed experiments. M J B, L E W analysed data and prepared figures. M J B, R B G and K A W wrote and edited the manuscript. R B G, L E W and K A W funded the study.

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