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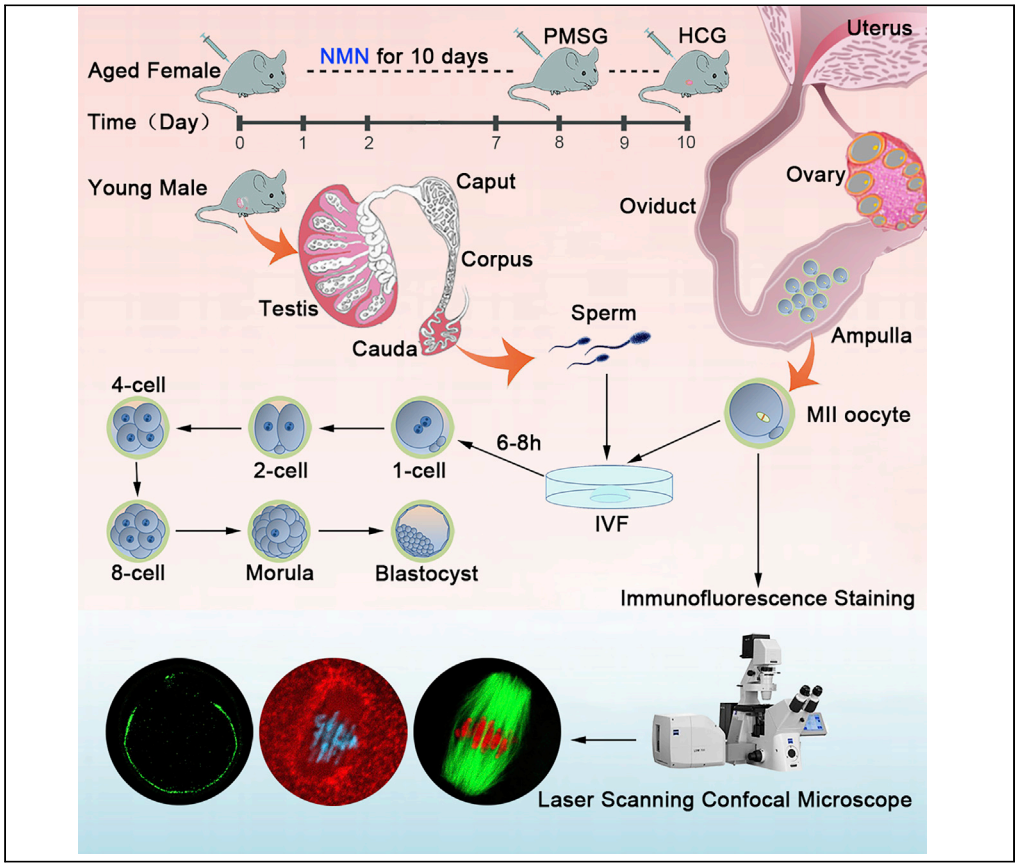
Protocol

Generation and assessment of high-quality mouse oocytes and embryos following nicotinamide mononucleotide administration



The low quality of oocytes is one of the main causes of the suboptimal reproductive outcome of female mammals with advanced maternal age. Here, we present a detailed protocol to obtain high-quality oocytes and embryos from aged mice by nicotinamide mononucleotide (NMN) administration. We also describe fluorescence staining procedures to assess the organelle dynamics in oocytes, and *in vitro* fertilization and embryo culture systems to evaluate the influence of NMN on the fertilization ability and embryonic development potential.

Yilong Miao, Jingyue Chen, Qian Gao, Bo Xiong

xiongbo@njau.edu.cn

HIGHLIGHTS

A protocol for harvest of high-quality mouse oocytes and embryos by NMN treatment

Fluorescence staining and confocal imaging to evaluate oocyte quality

In vitro fertilization and embryo culture procedures to assess embryo quality

Miao et al., STAR Protocols *2*, 100298
March 19, 2021
https://doi.org/10.1016/j.xpro.2021.100298

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Generation and assessment of high-quality mouse oocytes and embryos following nicotinamide mononucleotide administration

Yilong Miao,[1,3] Jingyue Chen,[1,3] Qian Gao,[2] and Bo Xiong[1,4,5,*]

[1]College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

[2]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: xiongbo@njau.edu.cn

https://doi.org/10.1016/j.xpro.2021.100298

SUMMARY

The low quality of oocytes is one of the main causes of the suboptimal reproductive outcome of female mammals with advanced maternal age. Here, we present a detailed protocol to obtain high-quality oocytes and embryos from aged mice by nicotinamide mononucleotide (NMN) administration. We also describe fluorescence staining procedures to assess the organelle dynamics in oocytes, and *in vitro* fertilization and embryo culture systems to evaluate the influence of NMN on the fertilization ability and embryonic development potential.

For complete information on the use and execution of this protocol, please refer to Miao et al. (2020).

BEFORE YOU BEGIN

Preparation of NMN solution

Timing: 10 min

1. Dissolve NMN in sterile PBS to prepare the solution at concentration of 100 mg/mL.

NMN administration and oocyte superovulation

Timing: 10 days

2. Intraperitoneally inject 14–16-month-old ICR female mice with 0.1 mL of PBS (control) or 100 mg/mL NMN (200 mg/kg body weight per day) using a 1 mL syringe with 20G needle for 10 consecutive days.

⚠ CRITICAL: The administration dose should be optimized by gradient test (0–1,000 mg/kg body weight) according to the age and strain of the mice. (See Troubleshooting 1)

3. At the 8th day, intraperitoneally inject female mice with 10 IU pregnant mare serum gonadotropin (PMSG).
4. After 44–48 h, intraperitoneally inject female mice with 10 IU human chorionic gonadotropin (hCG).

Note: 5 IU of PMSG and hCG are usually injected for superovulation in young mice. Aged mice are not sensitive to the gonadotrophins, and thus injection dose may need to be increased to 10 IU when necessary. (See Troubleshooting 2)

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-α-tubulin-FITC	Sigma-Aldrich	Cat# F2168; RRID: AB_2827403
Chemicals, peptides, and recombinant proteins		
Nicotinamide mononucleotide	Sigma-Aldrich	Cat# N3501
M2 medium	Sigma-Aldrich	Cat# M7167
M16 medium	Sigma-Aldrich	Cat# M7292
Albumin from bovine serum	Sigma-Aldrich	Cat# A1933
Mineral oil	Sigma-Aldrich	Cat# M8410
Triton X-100	Sigma-Aldrich	Cat# V900502
Tween 20	Sigma-Aldrich	Cat# V900548
Tyrode's solution, acidic	Sigma-Aldrich	Cat# T1788
Lens Culinaris Agglutinin (LCA)-FITC	Thermo Fisher Scientific	Cat# L32475
Phosphate-buffered saline	Thermo Fisher Scientific	Cat# 20012027
Hoechst 33342	Thermo Fisher Scientific	Cat# H3570
Propidium iodide	Thermo Fisher Scientific	Cat# P3566
EmbryoMax Human Tubal Fluid (HTF) medium	Millipore	Cat# MR-070-D
EmbryoMax KSOM medium	Millipore	Cat# MR-106-D
4% paraformaldehyde	Santa Cruz Biotechnology	Cat# 281692
Pregnant mare's serum gonadotropin (PMSG)	Jianchun (Nanjing, China)	Cat# A006
Human chorionic gonadotropin (HCG)	Jianchun (Nanjing, China)	Cat# A001-2
Critical commercial assays		
MitoTracker Red CMXRos	Thermo Fisher Scientific	Cat# M7512
Annexin V-FITC Apoptosis Detection Kit	Beyotime (Hangzhou, China)	Cat# C1062
Reactive Oxygen Species Detection Kit	Jiancheng (Nanjing, China)	Cat# E004
Experimental models: organisms/strains		
ICR mice	Animal Core Facility of Nanjing Medical University	N/A
Other		
Stereo microscope	Olympus	SZ-61
CO_2 incubator	Thermo Fisher Scientific	HERAcell 150i
Heating stage	CBL Photoelectron Technology	Model 100
Fine-tip forceps	Rhino	SW14
Glass slide	Citoglas	Cat# 7105P
35 mm Petri dish	Thermo Fisher Scientific	Cat# 121V
ProLong Gold antifade mountant	Thermo Fisher Scientific	Cat# P10144

MATERIALS AND EQUIPMENT

NMN solution

Reagent	Final concentration	Amount
NMN	100 mg/mL	1 g
Sterile PBS	n/a	10 mL

Note: Store at 4°C and warm to 20°C–25°C before use.

Gonadotrophins

PMSG is prepared as below.

Reagent	Final concentration	Amount
PMSG	5 IU/0.1 mL	5,000 IU
Sterile PBS	n/a	100 mL

Note: Store in single-use aliquots of 1 mL at −20°C and warm to 20°C–25°C before use.

hCG is prepared as below.

Reagent	Final concentration	Amount
hCG	5 IU/0.1 mL	10,000 IU
Sterile PBS	n/a	200 mL

Note: Store in single-use aliquots of 1 mL at −20°C and warm to 20°C–25°C before use.

Hyaluronidase solution

Reagent	Final concentration	Amount
Hyaluronidase	0.1%	10 mg
M2 medium	n/a	10 mL

Note: Store in single-use aliquots of 1 mL at −20°C and warm to 37°C before use.

Membrane-permeabilized solution (MPs)

Immunofluorescence (IF) MPs is prepared as below.

Reagent	Final concentration	Amount
10% Triton X-100	0.5%	0.5 mL
Sterile PBS	n/a	9.5 mL

Note: Store at 4°C and warm to 20°C–25°C before use.

Washing buffer

IF washing buffer is prepared as below.

Reagent	Final concentration	Amount
Tween 20	0.1%	10 μL
10% Triton X-100	0.01%	10 μL
Sterile PBS	n/a	9.98 mL

Dye staining washing buffer is prepared as below.

Reagent	Final concentration	Amount
BSA	0.1%	0.01 g
Sterile PBS	n/a	10 mL

Note: Store at 4°C and warm to 20°C–25°C before use.

Blocking buffer

IF blocking buffer is prepared as below.

Reagent	Final concentration	Amount
BSA	1%	0.1 g
Tween 20	0.1%	10 μL
10% Triton X-100	0.01%	10 μL
Sterile PBS	n/a	9.98 mL

Note: Store at 4°C and warm to 20°C–25°C before use.

Fixative

Fixative for chromosome spreading is prepared as below.

Reagent	Final concentration	Amount
4% paraformaldehyde	1%	250 μL
10% Triton X-100	0.15%	15 μL
Sterile PBS	n/a	735 μL

Note: Prepare just before use, and adjust pH to 9.2.

Human tubal fluid (HTF) medium

Two drops of 300 μL HTF for sperm capacitation and several drops of 50 μL HTF for fertilization are made in a dish with 35 mm diameter, covered with mineral oil and equilibrated in the incubator at 37°C with atmosphere of 5% CO_2 for 12–16 h before use (Figure 1).

Potassium simplex optimized medium (KSOM) medium

One drop of 50 μL KSOM for *in vitro* embryo culture is made in a dish with 35 mm diameter, covered with mineral oil and equilibrated in the incubator at 37°C with atmosphere of 5% CO_2 for 12–16 h before use.

Equipment

Turn on the stereo microscope and the heating stage (37°C) 5 min ahead of time. Prepare fine-tipped forceps, mouth pipette, and sterile alcohol cottons (Figure 2).

STEP-BY-STEP METHOD DETAILS

Collection of ovulated oocytes

Timing: 20 min

Before starting:

- Pre-warm M2 medium to 37°C before use.
- Prepare 1 mg/mL hyaluronidase with M2 medium in advance, and warm it up to 37°C for use.

1. Sacrifice the mice by cervical dislocation after 12–14 h of hCG injection.

⚠ CRITICAL: The timing to sacrifice the mice after hCG injection is important because it greatly affects the number of oocytes harvested from the oviduct.

2. Hold and cut the oviduct between ovary and uterus out of mice, and place it into M2 medium in the dish on the heating stage.

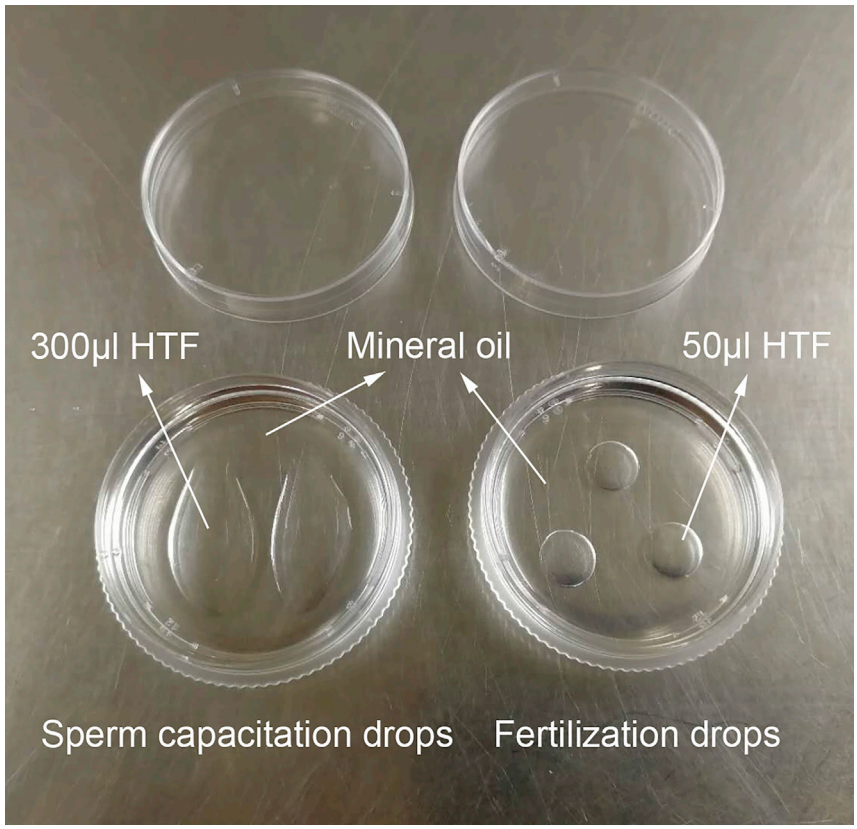

Figure 1. Preparation of media for sperm capacitation and fertilization
Two drops of 300 µL HTF for sperm capacitation and one drop of 50 µL HTF for fertilization are made in a dish with 35 mm diameter, covered with mineral oil.

3. Release ovulated oocytes in M2 medium by tearing a hole in the ampulla of the oviduct with fine-tipped forceps (Figure 3).

   Note: The ampulla is swollen and translucent such that the oocytes are visible. Be careful not to damage the oocytes by forceps. The heating stage (37°C) should be used to maintain temperature during oocyte manipulation.

4. Remove cumulus cells surrounding oocytes by brief incubation in 1 mg/mL hyaluronidase and then wash oocytes in fresh M2 medium on the heating stage.

   ⚠ CRITICAL: Do not exceed 3 min exposure to hyaluronidase as it will damage the oocytes.

Following oocyte collection and fertilization, the effect of NMN supplementation on oocyte and embryo quality can be assessed by 1) Antibody staining and confocal microscopy, go to step 5; 2) Dye staining and confocal microscopy, go to step 12; 3) Chromosome spreading, go to step 15; 4) Sperm binding assay, go to step 21; 5) *In vitro* fertilization and embryo culture, go to step 26.

Antibody staining and confocal microscopy

Timing: 1–1.5 days

5. Fix 20–30 oocytes in 4% paraformaldehyde (PFA)/PBS (pH 7.4) at 20°C–25°C for 30 min.

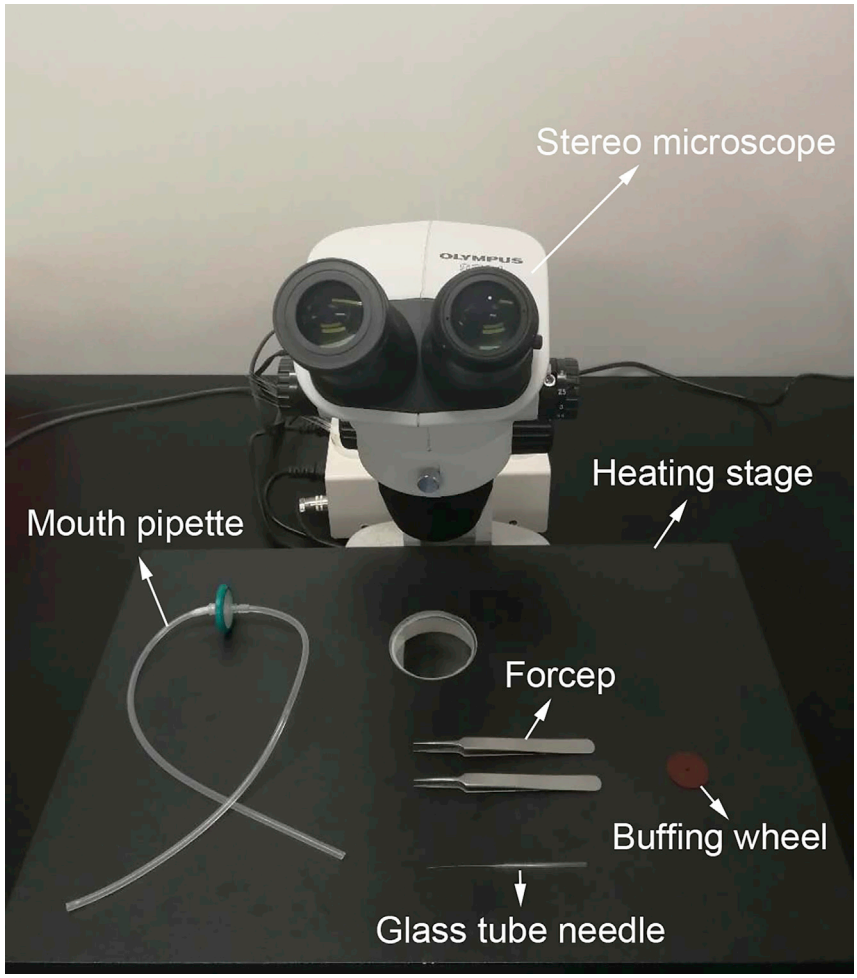

Figure 2. The equipment used for collection of mouse sperm and oocytes

Note: It is better to prepare the fixation solution just before use if needed.

6. Permeabilize oocytes in IF MPs at 20°C–25°C for 20 min.

   Note: Longer time of permeabilization will damage the oocytes.

7. Block oocytes in IF blocking buffer at 20°C–25°C for 1 h.

   Note: This step will reduce the background of the nonspecific binding of the primary antibody.

8. Incubate oocytes with primary antibodies in the blocking buffer at 4°C for 12–16 h.

   Alternatives: For some primary antibodies, oocytes can be incubated at 37°C for 4 h. In this case, the background of images might be higher.

   Note: The recommended antibody concentration: α-tubulin-FITC (1:200), LCA-FITC (1:100).

9. Wash oocytes in IF washing buffer, and then incubate them with the corresponding secondary antibodies at 20°C–25°C for 1 h.

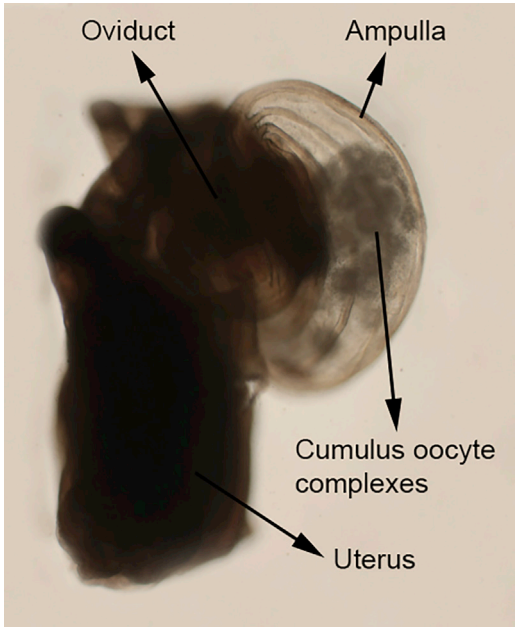

Figure 3. Representative image of cumulus-oocyte complexes (COCs) in the ampulla of oviduct

COCs can be seen in the swollen and translucent ampulla.

10. Counterstain oocytes with Hoechst 33342 (10 μg/mL) at 20°C–25°C for 10 min.
11. Mount 10 oocytes in 2 μL of antifade mountant on the microscope slides and seal by coverslip with lanolin. Acquire images under the laser scanning confocal microscope immediately after mounting or store the slide at −20°C.

Dye staining and confocal microscopy

Timing: 1.5–2 h

12. Incubate oocytes in M16 medium containing 500 nM cell permeant MitoTracker Red CMXRos, 2 μM MitoProbe JC-1, 10 μM dichlorofluorescein diacetate (DCFHDA), or 10% Annexin-V-FITC at 37°C with 5% CO_2 for 30 min for active mitochondrion staining, mitochondrial membrane potential assessment, DCFH staining, and Annexin-V staining, respectively.

Note: The process of dye staining should be completed in dark to avoid the fluorescence quenching.

13. Wash oocytes three times in 0.1% BSA/PBS.
14. Mount 10 oocytes in 2 μL of antifade mountant on the microscope slides and seal by coverslip with lanolin. Acquire images under the laser scanning confocal microscope immediately after mounting or store the slide at −20°C.

⚠ CRITICAL: For the measurement of fluorescence intensity, acquire images from both control and treated oocytes by following the same staining procedure and setting up the same imaging parameters of the confocal microscope. Apply Image J (NIH, Bethesda, MD, USA) to define a region of interest (ROI) in the images, and determine the average fluorescence intensity per unit area within the ROI. Compare the final average intensities between aged and NMN-supplemented groups.

Chromosome spreading

Timing: 1–1.5 h

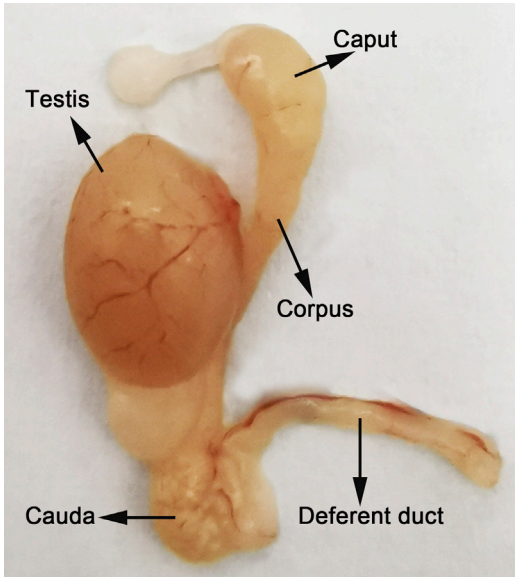

Figure 4. Representative image of male reproductive system in the mouse
Matured sperm can be collected from cauda epididymis.

15. Warm acidic Tyrode's solution (pH 2.5) and M2 medium to 37°C.
16. Transfer oocytes into 50 μL Tyrode's solution for 30 s to remove the zona pellucida (ZP).

Note: Too little exposure to the solution will not remove the ZP completely, which will prevent the oocytes from bursting and chromosomes from spreading. Too much exposure will damage and kill the oocytes. Watching the ZP dissolve under the dissection microscope can aid in optimizing the exposure time.

⚠ CRITICAL: Using fresh Tyrode's solution is critical, as its function declines with age. Use a fresh well of Tyrode's solution for each group of oocytes to ensure uniform digestion of the ZP. Tyrode's solution should be aliquoted and stored at −20°C.

17. Wash the oocytes by transferring into warmed M2 medium immediately after ZP removal.

⚠ CRITICAL: Be careful when transferring, as oocytes will easily stick together and to the glass pipette following removal of the ZP. Addition of 0.2% polyvinylpyrrolidone in the medium helps to prevent the oocytes from sticking.

18. Recover oocytes in M2 medium for 10 min at 37°C in a 5% CO2 incubator, and then transfer them with as little M2 medium as possible into one drop of fixative on the microscope slide.

Note: The transfer should be done within 30 s, using a microscope without a heating stage.

19. Observe oocyte dissolution under the microscope and leave the slide to air dry at 20°C–25°C for 30 min.
20. After air drying, stain the chromosomes with propidium iodide (PI) for 10 min and acquire images under the laser scanning confocal microscope.

Sperm binding assay

Timing: 2–2.5 h

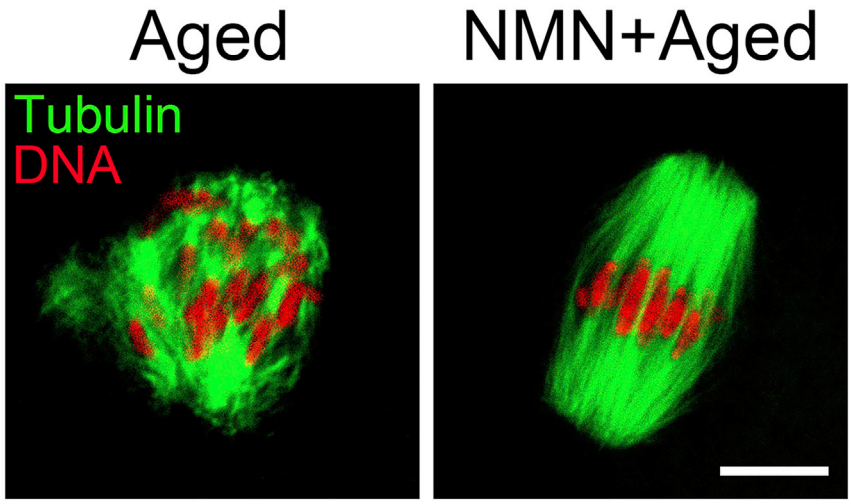

Figure 5. Representative images of spindle/chromosome structure in aged and NMN+aged oocytes
Metaphase I oocytes are immunostained with α-tubulin to show the spindle and counterstained with PI to display the chromosome. Scale bar, 15 μm.

Before starting:

- Equilibrate HTF in the incubator for 12–16 h before use.

21. Release sperm by slicing the cauda epididymides (Figure 4) from 3-month-old male mice (sacrificed by cervical dislocation) with fine-tipped forceps in HTF medium, followed by 1 h of capacitation at 37°C in a 5% CO_2 incubator.
22. Calculate sperm concentration by computer-assisted sperm analysis system, and add 4×10^5/mL capacitated sperm to 100 μL HTF containing 20–30 ovulated oocytes and 5 two-cell embryos at 37°C with 5% CO_2 for 30 min of binding.

Note: Prepare two-cell embryos as a negative wash control ahead of time, as the sperm cannot bind to embryos.

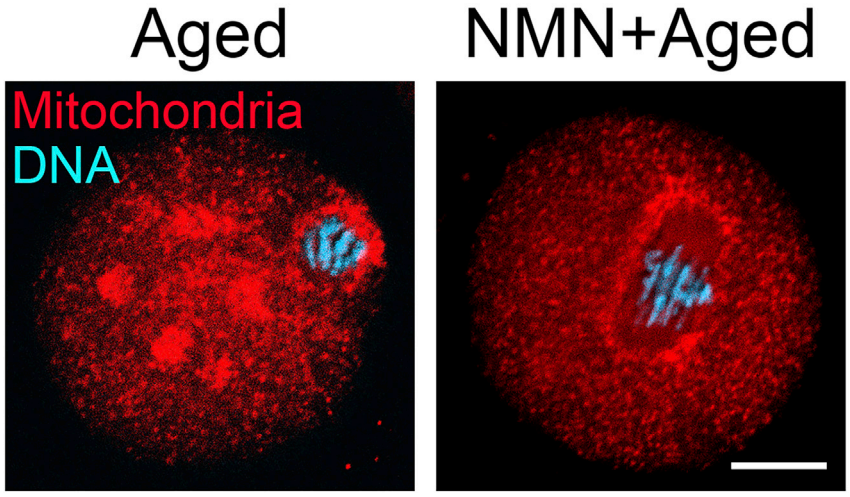

Figure 6. Representative images of mitochondrial distribution in aged and NMN+aged oocytes
Metaphase I oocytes are stained with MitoTracker Red to show the mitochondria and counterstained with Hoechst to display the chromosome. Scale bar, 20 μm.

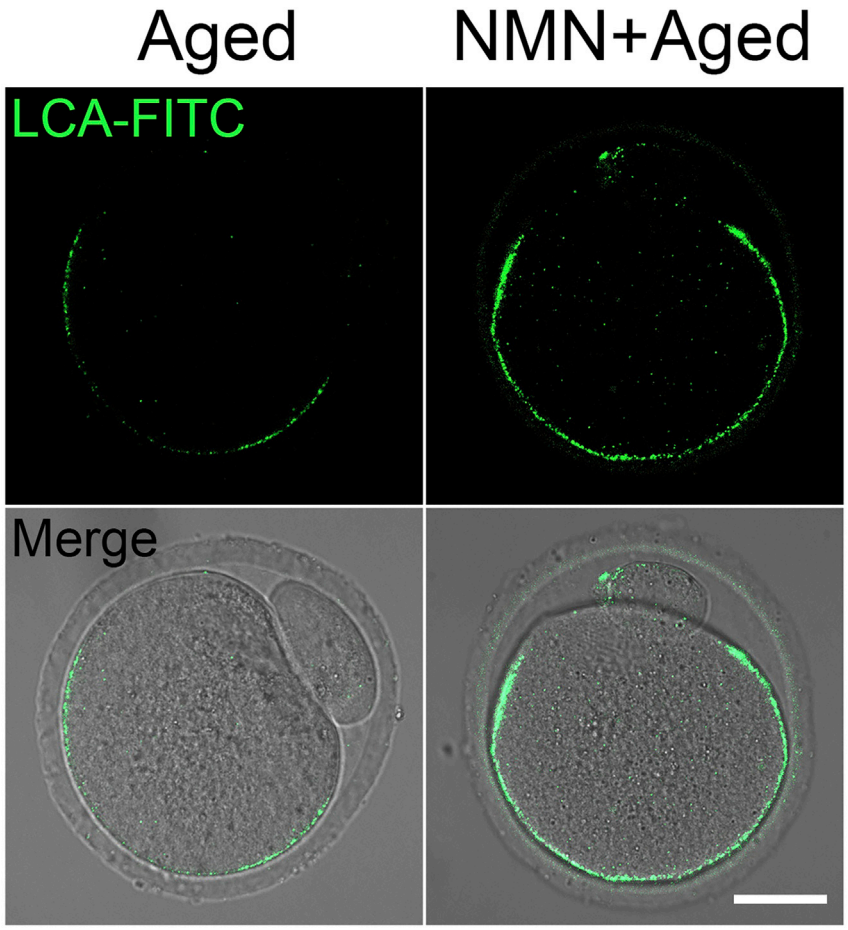

Figure 7. Representative images of cortical granule dynamics in aged and NMN+aged oocytes
Metaphase II oocytes are stained with LCA-FITC to show the cortical granules. Scale bar, 20 μm.

23. Wash sperm binding to the ovulated oocytes with a wide-bore pipette to remove all but 2–6 sperm on the two-cell embryos (negative control).

⚠ CRITICAL: Watch the sperm binding to the oocytes under the dissection microscope when washing them. Too much or too little wash will affect the number of sperm binding to the oocytes.

24. Fix the oocytes with sperm in 4% PFA for 30 min, and stain them with Hoechst 33342 for 10 min.
25. Quantify the bound sperm from z projections acquired by confocal microscope.

In vitro fertilization and embryo culture

Timing: 5 days

Before starting:

- Equilibrate HTF in the incubator for 12–16 h before use.
- Equilibrate KSOM in the incubator for 12–16 h before use.

26. Release sperm by slicing the cauda epididymides from 3-month-old male mice with fine-tipped forceps in HTF medium, followed by 1 h of capacitation at 37°C in a 5% CO_2 incubator.

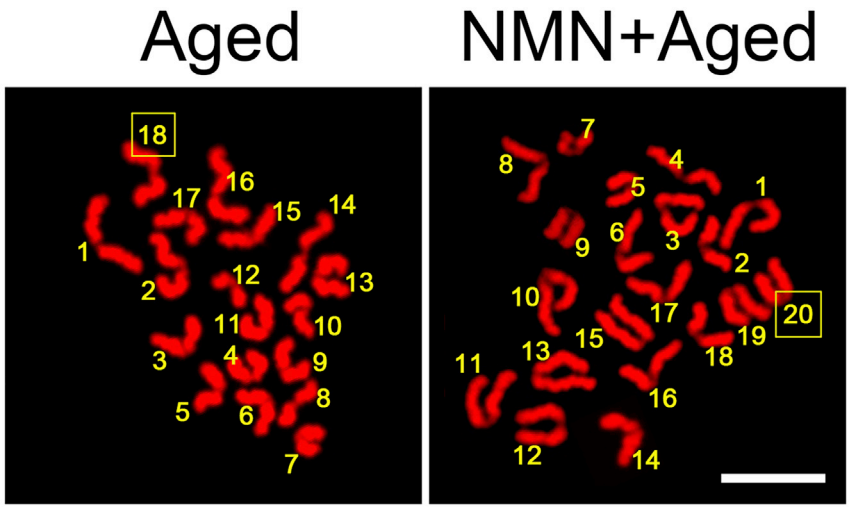

Figure 8. Representative images of chromosome euploidy in aged and NMN+aged oocytes
Chromosome spreading is performed to count the number of chromosomes. Scale bar, 5 μm.

27. Add 4 × 10^5/mL capacitated sperm to 100 μL HTF containing 20–30 ovulated oocytes at 37°C with 5% CO_2 for 5 h of incubation (Methods Video S1).

Note: The presence of two pronuclei is scored as successful fertilization.

⚠ CRITICAL: Observe the activity of sperm before addition to the oocytes, as it is critical for the success of the fertilization. If the sperm activity is weak, release the sperm from another male mouse immediately. (See Troubleshooting 3)

28. Transfer the fertilized oocytes to a 96-well culture plate containing 100 μL KSOM medium covered with mineral oil at 37°C in a 5% CO_2 atmosphere for *in vitro* embryo culture for 4 days to the blastocysts.

⚠ CRITICAL: Do not frequently (more than once per day) take embryos out of the incubator to observe the development during the culture, as it will greatly impact the progression of embryonic development. (See Troubleshooting 4)

EXPECTED OUTCOMES

The advanced maternal age of female mammals is highly correlated to the decline of oocyte quality, which determines the reproductive performance of females (Balkenende et al., 2020; Deng, 2012; Traub and Santoro, 2010). The strategies to improve the oocyte quality and reproductive performance of females have been a hot research topic in the area of reproductive biology and medicine. Nicotinamide adenine dinucleotide (NAD^+) is a coenzyme of many enzymes (Gomes et al., 2013; Yoshino et al., 2018; Yoshino et al., 2011), especially the Sirtuin family proteins and it has been implicated in a variety of biological processes such as DNA damage repair, autophagy, adaptive stress responses, genomic stability, and cell survival (Bonkowski and Sinclair, 2016; Croteau et al., 2017; Kennedy et al., 2016). Of note, the decreased NAD^+ levels with age across many tissues are involved in the aging-related diseases (Bonkowski and Sinclair, 2016; Das et al., 2018; de Picciotto et al., 2016). Therefore, as a precursor metabolic intermediate of NAD^+, supplementation of NMN is an effective way to recover the NAD^+ levels and it is likely to be a feasible anti-aging intervention.

Here, we present an efficient protocol to generate high-quality oocytes from the aged mice by *in vivo* supplementation of NMN. In this way, a higher number of ovulated oocytes with reduced

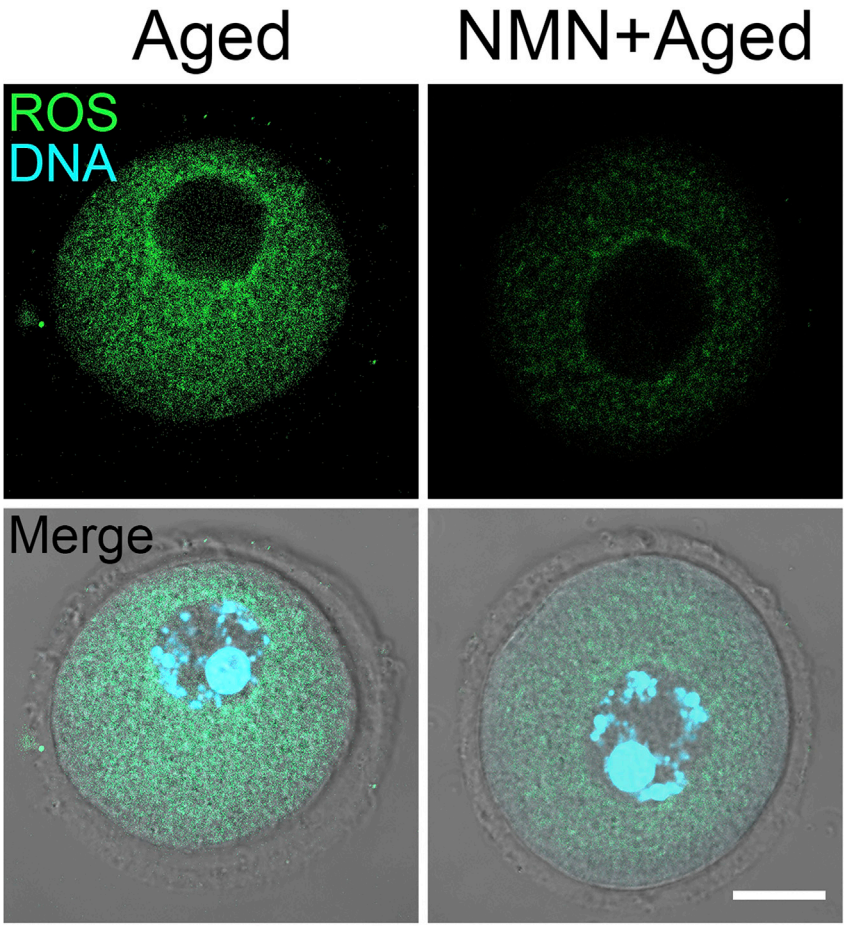

Figure 9. Representative images of ROS signals in aged and NMN+aged oocytes
Oocytes are stained with DCFH to show the ROS signals and countered with Hoechst to display DNA. Scale bar, 20 μm.

fragmentation of cytoplasm can be obtained. By fluorescence staining and imaging, key indicators of the oocytes, such as the spindle/chromosome structure, mitochondrial distribution, cortical granule dynamics, and chromosome ploidy, can be assessed. In aged oocytes, a higher frequency of disorganized spindle apparatus with misaligned chromosomes (Figure 5), aggregated clusters of mitochondria in the cytoplasm (Figure 6), discontinuous distribution of cortical granules in the cortex (Figure 7) and more or less number of chromosomes (Figure 8) will be observed. NMN supplementation is expected to restore these defects in aged oocytes (Figures 5, 6, 7, and 8). Additional parameters of oocyte health, specifically reduced level of ROS (Figure 9) and limited occurrence of apoptosis (Figure 10), in NMN-supplemented oocytes can also be evaluated. Moreover, since the decline of oocyte quality is reflected in the impairment of fertilization ability as well (Miao et al., 2018), the sperm binding assay and *in vitro* fertilization assay can be used to monitor the increased number of sperm binding to the oocytes and elevated fertilization rate in NMN-supplemented oocytes. Lastly, subsequent *in vitro* embryo culture is expected to further demonstrate the enhanced early embryonic development potential of NMN-supplemented oocytes (Figure 11).

LIMITATIONS

This protocol only describes an *in vivo* method by NMN injection to improve the oocyte and embryo quality from aged mice. If the researchers are planning to administrate NMN by drinking water or gavage, the optimal dose and time of NMN supplementation should be tested, because the high

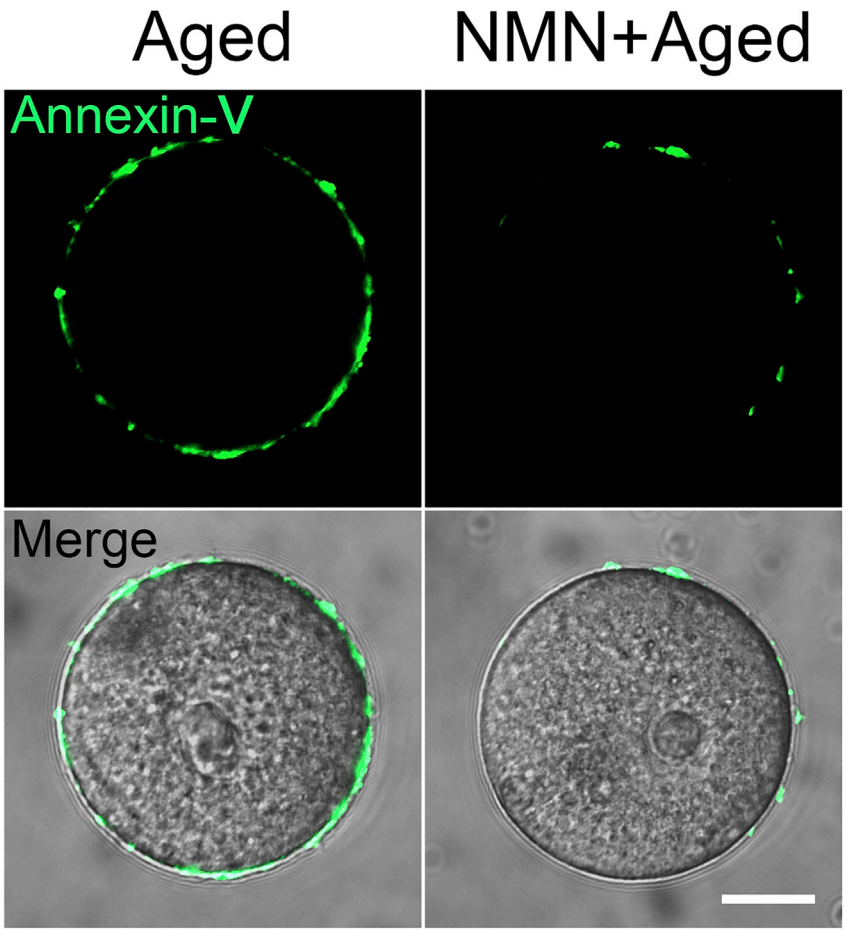

Figure 10. Representative images of apoptotic oocytes in aged and NMN+aged groups
Annexin-V is stained to indicate the apoptotic status of oocytes. Scale bar, 20 μm.

dose of NMN will negatively affect the oocyte and embryo quality. NMN could also be supplemented to the culture medium to promote the oocyte maturation and embryonic development *in vitro*, the concentration should be optimized.

TROUBLESHOOTING

Problem 1

Failure to produce positive effects after NMN supplementation

Potential solution

The dose of NMN is critical for its impact on the oocyte and embryo quality. Administration of low dose probably has no beneficial effects, but administration of high dose might result in adverse outcomes. Thus, it is necessary to determine the optimal administration dose and time according to the age and strain of the mice. Furthermore, different administration methods, such as intraperitoneal injection, intravenous injection, gavage, and water feeding, might have great divergences in effective doses.

Problem 2

Failure to harvest ovulated oocytes from aged mice

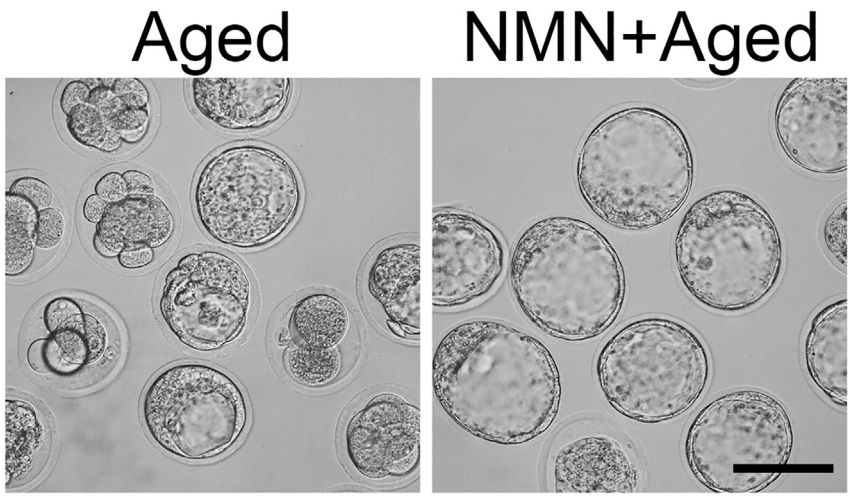

Figure 11. Representative image of blastocysts developed from fertilized aged and NMN+aged oocytes
Aged or NMN+aged oocytes are fertilized by normal sperm *in vitro* and then cultured in KSOM for embryonic development. Scale bar, 80 μm.

Potential solution

Given that less matured oocytes can be produced in aged mice, as well as the insensitivity of aged mice to the gonadotrophins, higher doses of PMSG and hCG might be needed for maximum superovulation of oocytes. Besides, make sure to collect the oocytes 12–14 h after hCG injection, the inappropriate timing would decrease the number of superovulated oocytes.

Problem 3

Low fertilization rate

Potential solution

The sperm should be released from male mice with the age of at least 10-week-old, otherwise it always leads to the impaired fertilization. The capacitation step is also critical for sperm to acquire the fertilizing ability. Afterwards make sure the sperm swim progressively and that the number of sperm used for fertilization is optimal (2–4 × 10^5/mL). If dead or motionless sperm are clustered over a half, replace by another male mouse. In addition, the ovulated oocytes should be collected just before use, as the long time incubation in the medium will result in the postovulatory aging and decreased fertilization ability of the oocytes.

Problem 4

Low embryonic development rate

Potential solution

Do not take the embryos out of the incubator frequently to observe the developmental status during *in vitro* culture, as it will maximize the ambient air exposure to damage the embryos. In addition, the shelf life of the culture medium KSOM is short, make sure to use it prior to expiration.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources can be directed to the Lead Contact, Bo Xiong (xiongbo@njau.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100298.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (2018YFC1003802, 2018YFC1004002), National Natural Science Foundation of China (31900592), Natural Science Foundation of Jiangsu Province (BK20190526), and China Postdoctoral Science Foundation (2019M651849).

AUTHOR CONTRIBUTIONS

B.X. conceived, designed, and supervised the study; Y.M., J.C., and Q.G. conducted the experiments; Y.M. and B.X. analyzed the data; Y.M. and B.X. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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