

NMN-SIRT1 signaling ameliorates IUGR via oxidative stress-mediated mitophagy to promote placental angiogenesis

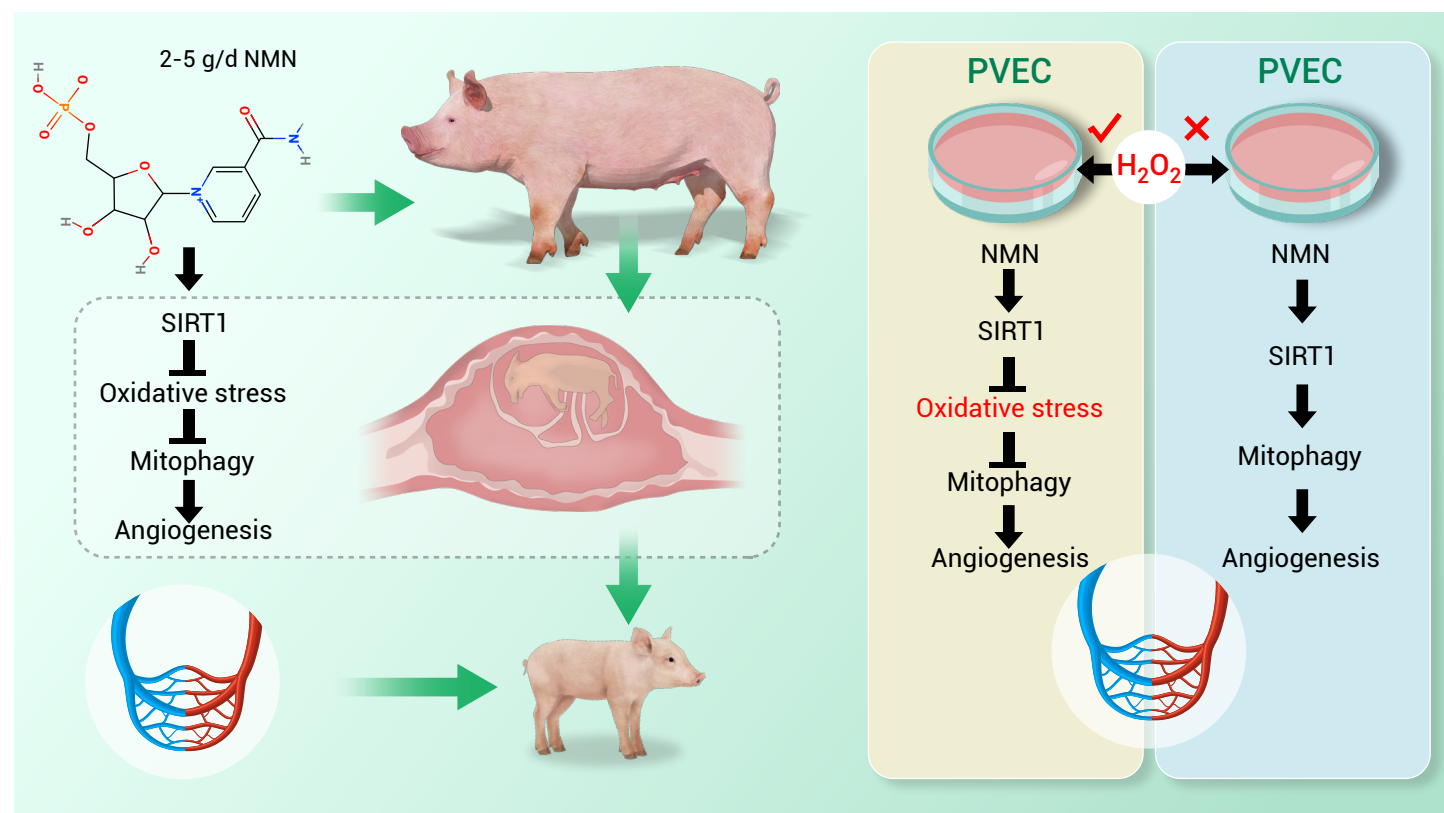
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Dietary NMN (2-5g/d) enhances placental angiogenesis and reduces IUGR incidence in sows.
- NMN upregulates placental SIRT1, alleviating oxidative stress in sows/offspring.
- NMN-SIRT1 axis inhibits excessive mitophagy and promotes angiogenesis in placenta.
- NMN-SIRT1 activation restores PVEC migration via oxidative stress/mitophagy regulation.
- Baseline NMN-SIRT1 signaling stimulates mitophagy to sustain PVEC angiogenic capacity.

NMN-SIRT1 signaling ameliorates IUGR via oxidative stress-mediated mitophagy to promote placental angiogenesis

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Nicotinamide mononucleotide (NMN) is a natural anti-aging agent. However, its effects on intrauterine growth restriction (IUGR) remain insufficiently explored. This study found that daily intake of 2–5 g/d of NMN significantly enhanced placental angiogenesis and reduced the incidence of IUGR by using a pregnant sow model (Landrace × Yorkshire breed). Mechanistically, NMN upregulated SIRT1 expression in the placenta, alleviated oxidative stress in sows, placentas, and their offspring, inhibited excessive mitophagy in the placenta, and promoted angiogenesis. Furthermore, activation of the NMN-SIRT1 signaling pathway was observed to mitigate oxidative stress and mitophagy, thereby restoring the migratory capacity of porcine vascular endothelial cells (PVECs) and improving endothelial function. However, in the absence of oxidative stress, NMN-SIRT1 acts as a potent mitophagy-promoting signal, positively regulating PVEC angiogenesis. In conclusion, our study demonstrates that the activation of NMN-SIRT1 signaling is crucial for reducing adverse pregnancy outcomes by ameliorating placental dysfunction induced by perinatal oxidative stress.

INTRODUCTION

Intrauterine growth restriction (IUGR) is a prevalent complication during pregnancy, typically characterized by impaired or slowed growth and development of the fetus or its organs within the uterus.^{1,2} IUGR is an important cause of fetal, perinatal and neonatal morbidity and mortality.³ The most common causes of mortality are severe placental insufficiency and chronic hypoxia, congenital malformations, congenital infections, placental abruption, cord accidents, cord prolapse, placental infarcts, and severe perinatal depression.⁴ IUGR is defined as a birth weight that is 2 standard deviations below the 10th percentile of normal weight for a child of the same gestational age.⁵ The incidence of IUGR varies from 4–8% in industrialized countries to 30% in less developed countries.⁶ However, the underlying causes differ significantly between regions. In developing countries, IUGR is predominantly associated with maternal malnutrition and infectious diseases, whereas in developed countries, it is more frequently linked to maternal conditions such as eating disorders, chronic diseases, and lifestyle factors including smoking, alcoholism, or drug abuse during pregnancy.^{7,8} Additionally, IUGR is often associated with factors such as chromosomal abnormalities and placental abnormalities.⁹ The placenta functions as the sole medium for material transfer between the mother and fetus. The initiation, maturation, and maintenance of the placental vascular system are crucial for a successful pregnancy.^{10–14} Previous studies on IUGR placenta suggest that the occurrence of IUGR may be associated with reduced placental blood flow and impaired angiogenesis at the maternal-fetal interface. The development of terminal villi in IUGR placentas is significantly reduced compared to normal full-term placentas.^{15–17} The sow is a polytocous animal, with up to 20–30% of piglets in commercial pig farms weighing less than 1.0 kg at birth, making it an excellent model for studying IUGR.^{18,19} Recent research also demonstrated that in sows, IUGR

piglets have significantly fewer placental blood vessels than piglets with normal birth weights.²⁰ Additionally, placental blood vessel density is highly positively correlated with piglet birth weight, indicating that restricted placental angiogenesis is a key factor contributing to the decrease in piglet birth weight.²¹ Incorporating nutrients that promote placental angiogenesis—such as arginine, methionine, cysteine, and allicin—into the diets of pregnant sows has been shown to effectively reduce the incidence of IUGR.^{21–25} This underscores the essential role of proper placental angiogenesis regulation in influencing fetal survival, development, and favorable pregnancy outcomes.

Oxidative stress is a key contributor to adverse pregnancy outcomes.²⁶ During pregnancy, particularly in the later stages and perinatal period, maternal oxidative stress progressively increases. As the fetus undergoes rapid development, the mother enters a catabolic state, which exacerbates insulin resistance and elevates oxidative stress levels.^{27–29} Excessive oxidative stress can impair both maternal and placental functions, disrupting placental blood flow, reducing the exchange area of placental microvilli, and decreasing placental perfusion. These conditions maintain the uterine placental microcirculation in a state of high resistance, negatively affecting the exchange of substances between the mother and fetus, potentially hindering fetal development and even leading to miscarriage.^{26,30–32} Peng et al. analyzed the serum and placenta of sows on the 90th day of pregnancy and at the time of delivery. They found that during the late stage of pregnancy, the body of sow experiences elevated oxidative stress, resulting in significantly reduced ATP levels, mtDNA content, and mitochondrial respiratory chain complex I activity in the placenta.³³ Similarly, Hu et al. reported increased reactive oxygen species (ROS) levels and decreased mitochondrial ATP production in the placenta of IUGR piglets.²⁰ Furthermore, during pregnancy, obesity exacerbates damage to the antioxidant defense system of sow, leading to excessive ROS production and a subsequent rise in the incidence of IUGR.³⁴ Zhou et al.³⁵ found that, compared to sows with appropriate backfat levels, obese pregnant sows exhibited significantly elevated levels of ROS and malondialdehyde (MDA) in their plasma. This increase was associated with elevated inflammatory factors in the placenta and a marked reduction in placental vascular density, further contributing to the incidence of IUGR in piglets. While numerous studies have demonstrated that oxidative stress negatively affects placental angiogenesis, the molecular mechanisms by which oxidative stress inhibits this process remain incompletely understood.

Angiogenesis is a dynamic and energy-consuming process, and optimal mitochondrial function is essential for facilitating angiogenesis.^{20,36} Nicotinamide mononucleotide (NMN) acts as a precursor for Nicotinamide Adenine Dinucleotide (NAD⁺) biosynthesis and is known for promoting cellular NAD⁺ production and enhancing mitochondrial function, which may help delay age-related diseases.^{37–42} Specifically, models of DNA damage repair and neurodegenerative diseases indicate that decreased NAD⁺ levels lead to reduced autophagic flux. Conversely, NMN can regulate both autophagy and mitophagy through Sirtuins, primarily Silent Information Regulator 1 (SIRT1),

thereby improving oxidative stress levels in tissues and organs.^{37,39,43} Xeroderma pigmentosum group A (XPA), a severe neurodegenerative disorder, is characterized by deficiencies in nucleotide excision repair. XPA-deficient cells exhibit mitophagy deficiency, with reduced activity of the NAD⁺-SIRT1-PGC1 α axis being a significant contributing factor. Supplementation with NMN can restore mitophagy and support mitochondrial health.⁴⁴ The occurrence of mitophagy is highly dependent on tissue type, energy levels, stress, and environmental signals, serving as an effective protective mechanism under stress or abnormal conditions.^{45,46} Various pregnancy-related disorders in humans, such as preeclampsia and IUGR, may be linked to mitophagy dysfunctions in the placenta, including elevated mitochondrial ROS levels and reduced placental blood perfusion.^{47,48} These studies highlight a critical connection between placental mitophagy and angiogenesis. However, research on the role and mechanisms of NMN in placental angiogenesis in sows remains limited. Therefore, this study aims to investigate the effects of NMN on reproductive performance, placental vascular development, and mitophagy in perinatal sows through in vivo and in vitro experiments. This research uncovers how NMN regulates placental angiogenesis in sows and provides new nutritional regulatory targets to reduce the incidence of IUGR in piglets.

MATERIALS AND METHODS

Experimental animals

In this study, a total of 60 primiparous sows of the Landrace $\times$ Yorkshire breed, matched for age and weight, were selected and randomly assigned to three treatment groups, each consisting of 20 sows with one sow per replicate. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee of Zhongkai University of Agriculture and Engineering (Ethical approval number, ZHKU-2023-0220). Since no studies have yet examined the dosage of NMN administered to gestating sows, we calculated a dose range of 1.80 g/d to 6.48 g/d. This calculation was based on the formula proposed by Nair A. B. et al.⁴⁹ for converting drug doses across mammalian species and referenced the range of NMN doses used in human studies.^{50,51} Therefore, in the present study, the control group (NC) received a standard pregnancy diet, while experimental group 1 (2 g/d) was supplemented with 2 g/d of NMN in addition to the standard diet, and experimental group 2 (5 g/d) received 5 g/d of NMN alongside the standard diet. The duration of the experiment was 44 days, commencing on the 70th day of gestation and concluding at the end of delivery on the 114th day of gestation. All experimental sows were synchronized in estrus, mating, and delivery.

Determination of reproductive performance of sows

On the day of sow delivery, record the number of total born piglets, live births, normal-birth-weight piglets, low-birth-weight piglets, mummified fetuses, and stillborn piglets. Additionally, calculate the rate of low-birth-weight piglets. Simultaneously, weigh the newborn piglets and the placentas, and calculate the piglet litter weight. Piglets with a birth weight $\leq$ 0.97 kg are classified as weak piglets.

Determination of biochemical indicators in sow plasma, placenta, and piglet plasma

The determination of biochemical indicators in sow plasma, placenta, and piglet plasma includes total cholesterol (T-CHO), total protein (TP), urea nitrogen (BUN), albumin (ALB), glucose (GLU), total antioxidant capacity (T-AOC), MDA, hydrogen peroxide (H₂O₂), catalase (CAT), superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px). The test kits used in the experiment were procured from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China), and the measurement process adhered strictly to the experimental protocols.

Placental hematoxylin-eosin (H&E) and immunofluorescence staining (IF)

Collect placental tissue from the same location and store it in 4% paraformaldehyde. Subsequently, embed the tissue in paraffin and slice it into sections. Stain the sections with hematoxylin and eosin (H & E). Place the stained sections under a microscope and capture images, randomly selecting three fields of view for each section. Utilize ImageJ to measure the placental vascular area and calculate the vascular density. Placental vessel density = total vessel area of stroma / stroma area $\times$ 100%.⁵² The immunoflu-

orescence procedure for placental tissue is based on the methodology described by Kasikova et al.⁵³ This process involves embedding placental tissue fixed in 4% paraformaldehyde in paraffin, with a section thickness of 5 mm. After dewaxing, antigen retrieval, and blocking, the tissue is incubated with primary antibodies and fluorescent secondary antibodies, followed by nuclear staining with DAPI (Beyotime, Shanghai, China). The fluorescence intensity of CD31 and Parkin in the placental tissue is detected and photographed using a fluorescence inverted microscope. The fluorescence intensity is quantified using ImageJ software.

Cell culture

Porcine vascular endothelial cells (PVECs) utilized in this experiment were obtained from the cell bank of the Chinese Academy of Sciences (GNO15, Shanghai, China). The PVECs were cultured in RPMI 1640 medium (Sangon, Beijing, China) supplemented with 10% fetal bovine serum (TransGen, Beijing, China) and were maintained under the following incubator conditions: 37°C, 5% CO₂, and saturated humidity.

RT-qPCR

RNA was extracted from placental tissue and PVEC cells following the instructions provided in the Trizol assay kit (TransGen, Beijing, China). The concentration of RNA was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA), and RNA samples with a total concentration of 3 μ g were reverse transcribed to synthesize cDNA. The mRNA expression levels of antioxidant-related genes, mitochondrial autophagy-related genes, and angiogenesis-related genes were assessed using real-time quantitative PCR (RT-qPCR). The PCR reactions were conducted on the CFX real-time quantitative PCR detection system (Bio-Rad, USA). B2M1 was selected as the reference gene for placental tissue normalization, while β -actin was chosen as the reference gene for intracellular normalization. The relative expression levels were calculated using the 2^{- $\Delta\Delta$ Ct} method.⁵⁴ The primer sequences for each target gene were designed using NCBI primer design software and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences utilized in the experiment are shown in Table S1.

Western blot

Referring to the previous method,²⁴ the total protein content of placental tissues and PVEC cells was determined using the BCA protein concentration assay kit (Beyotime, P0011). ImageJ software was employed to perform grayscale analysis on the protein bands, using β -actin as an internal reference for normalization to evaluate the expression levels of the target proteins. The details of the protein antibodies used in the experiment are shown in Table S2.

Statistical analysis

After the original data were recorded and processed using Excel, SPSS 28 software was employed to conduct a one-way ANOVA. The normality of the data was assessed using the Shapiro-Wilk test, while the homogeneity of variance was confirmed through the Levene test, ensuring that all data met the assumptions required for parametric testing. Subsequently, differences between groups were analyzed using One-way ANOVA, followed by multiple comparisons conducted with the Tukey HSD method. The image data were similarly analyzed using one-way ANOVA with GraphPad Prism 9.0.0 software and were further compared using a Tukey HSD method for multiple comparisons, with * denoting $P < 0.05$ and ** denoting $P < 0.01$. The Pearson correlation coefficient method was utilized to calculate correlations. The data in the graphs are presented as "mean $\pm$ standard error (SEM)".

RESULTS

NMN reduces the birth rate of IUGR piglets and alleviates oxidative stress in pregnant sows and their offspring

This study investigated the effects of different doses of NMN on the gestational diet of sows, starting from the 70th day of pregnancy. After giving birth (114th day of pregnancy), the weights of the piglets and placentas were recorded and analyzed statistically. The results indicated that, compared to the control group (NC), adding 2 g/d of NMN significantly increased the

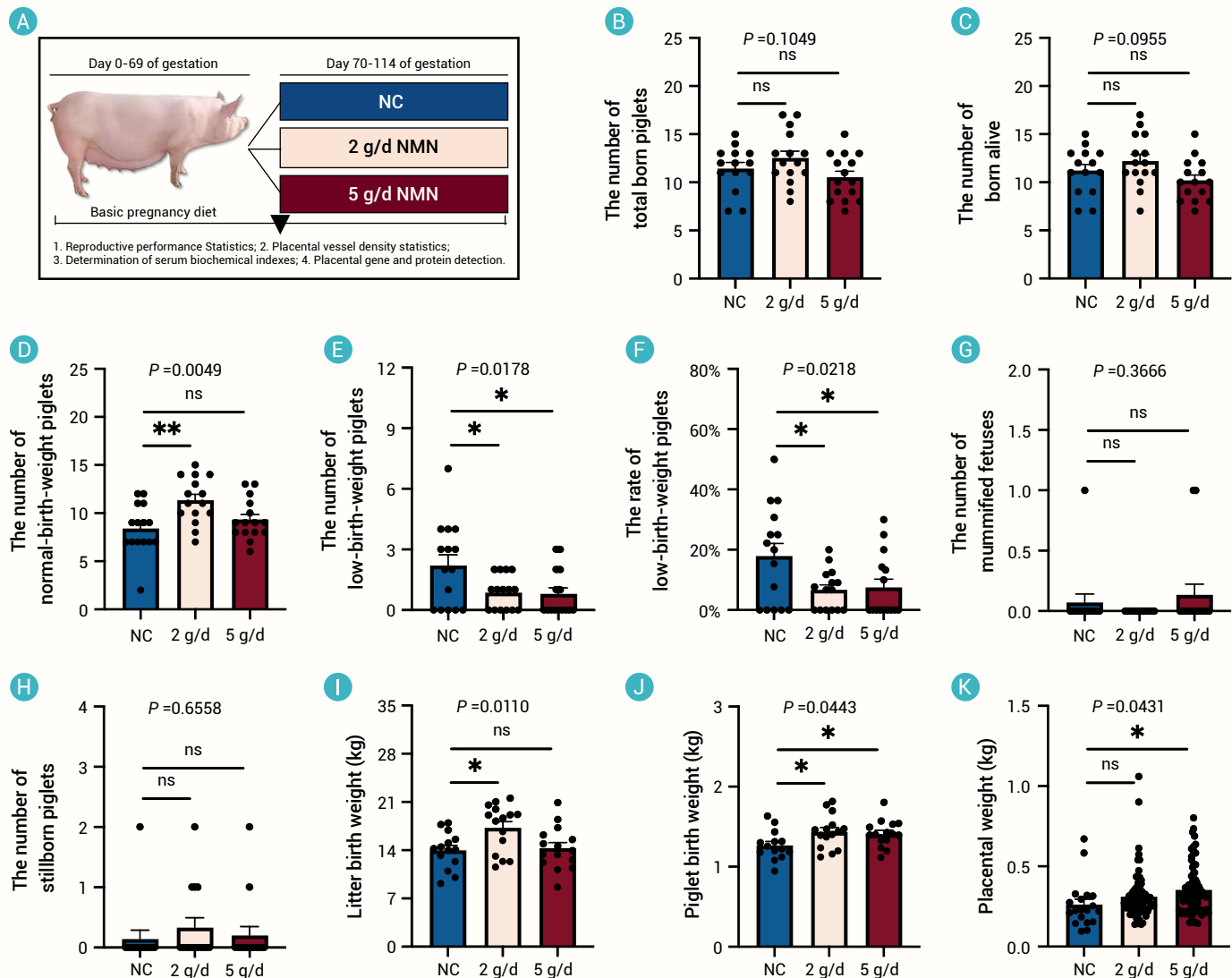


Figure 1. Effects of NMN supplementation on reproductive performance of sows during pregnancy (A) Schematic diagram of sow experimental grouping. (B-E, G, H) The number of total born piglets, born alive, normal-birth-weight piglets, low-birth-weight piglets, mummified fetuses, stillborn piglets of sows. (F) The rate of low-birth-weight piglets. (I) The litter birth weight of sows. (J) The piglet birth weight of sows. (K) The placental weight of sows. * denoting $P < 0.05$ and ** denoting $P < 0.01$. Data are means $\pm$ SEM, $n=15/\text{group}$.

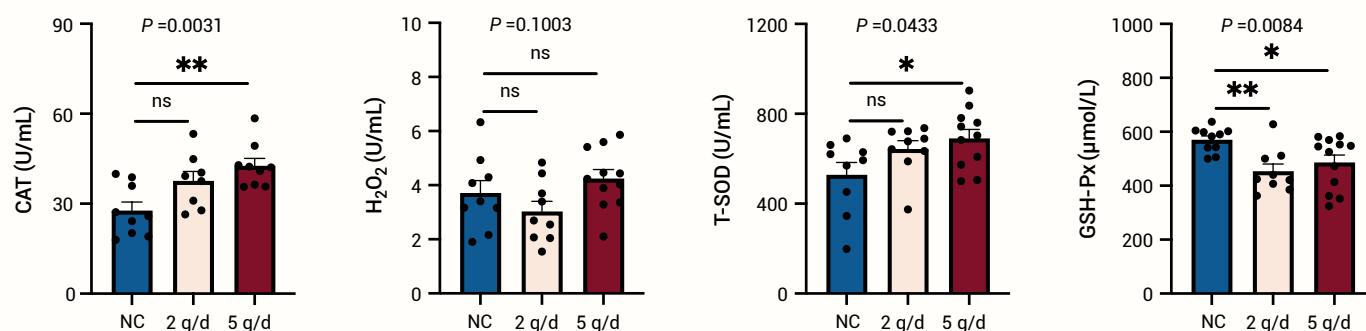
number of normal-birth-weight piglets, as well as the total litter birth weight and the average weight of the piglets. Furthermore, the addition of 5 g/d of NMN significantly enhanced both the average birth weight and placenta weight of the piglets. Incorporating NMN into the pregnancy diet also significantly reduced both the number and proportion of low-birth-weight piglets produced by sows (Figure 1). These findings suggest that supplementing the pregnancy diet with NMN can markedly improve the reproductive performance of sows and decrease the incidence of low-birth-weight piglets.

On the 114th day of pregnancy (the day of delivery), blood was collected from the ear veins of sows, and plasma was extracted to measure biochemical indicators of oxidative stress, antioxidant activity, and glucose metabolism. The experimental results indicated that, compared to the NC group, supplementing the pregnancy diet with 2 g/d of NMN significantly increased plasma CAT activity in sows. Furthermore, the addition of 5 g/d of NMN to the pregnancy diet significantly enhanced both plasma CAT and T-SOD activity in sows, although NMN significantly reduced GSH-Px activity (Figure 2A). These findings suggest that incorporating NMN into the gestational diet alleviates oxidative stress in sows during the perinatal period. Additionally, the experimental results revealed that, compared to the NC group, the addition of 2 g/d NMN to the pregnancy diet significantly increased the T-

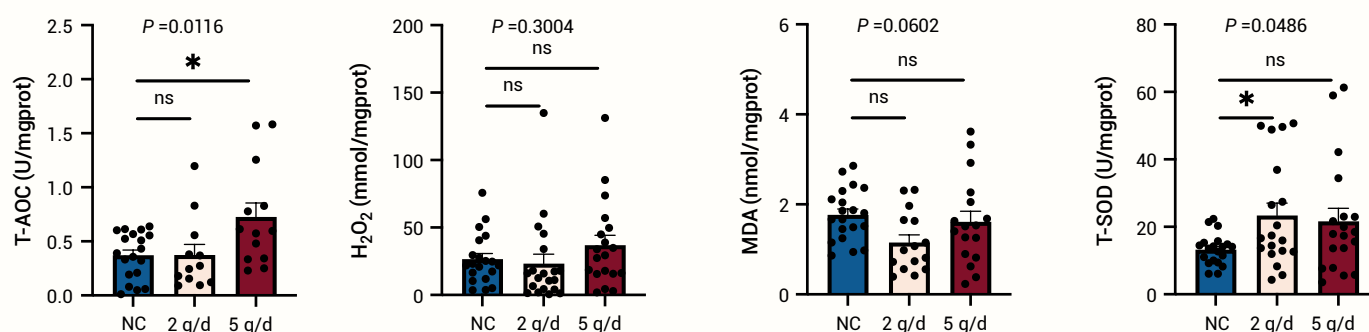
SOD activity of the placenta. Supplementing with 5 g/d of NMN significantly improved the T-AOC ability of the placenta (Figure 2B). Moreover, 2 g/d NMN supplementation significantly elevated placental GLU content, while the addition of 5 g/d NMN significantly increased placental TP content. Furthermore, adding NMN into the pregnancy diet significantly reduced placental BUN content (Figure S1B). These findings suggest that adding NMN to pregnancy diets enhances the antioxidant capacity of the placenta and promotes nutrient metabolism and transport.

On the first day after birth, blood samples were collected from the anterior vena cava of piglets, and the upper plasma layer was extracted for biochemical index determination of oxidative stress and antioxidant activity. The results indicated that, compared to the NC group, the addition of NMN to the pregnancy diet significantly increased the activities of CAT, T-SOD, and GSH-Px in piglet plasma, while also significantly decreasing MDA content (Figure 2C). Moreover, supplementing the pregnancy diet with 2 g/d NMN significantly increased the plasma T-CHO level in piglets, while significantly decreasing the plasma BUN level (Figure S1C). These findings suggest that adding NMN into the gestational diet enhances the antioxidant capacity and improves glucose and lipid metabolism in piglets.

A Sow's plasma



B Placenta



C Piglet's plasma

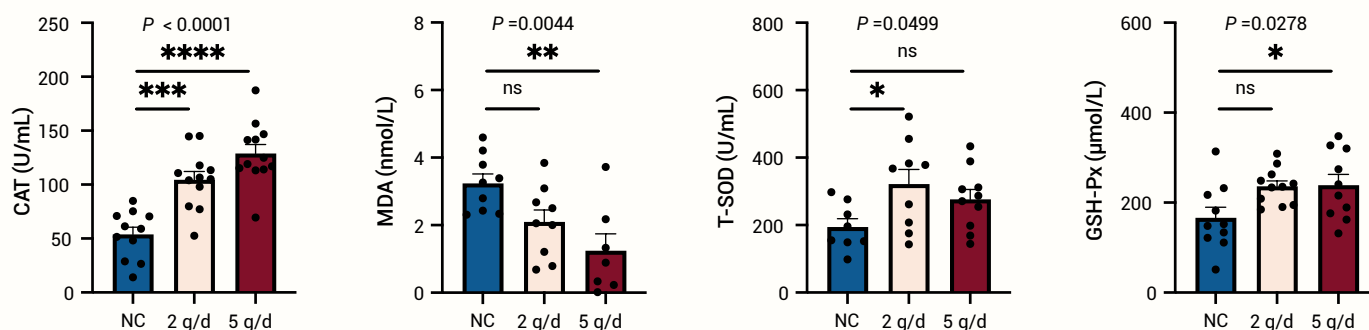


Figure 2. Effects of NMN supplementation on oxidative stress in sows, placentas and piglets (A) The content of CAT, H₂O₂, T-SOD and GSH-Px in sow's plasma (n=12/group). (B) The content of T-AOC, H₂O₂, MDA and T-SOD in placenta (n=20/group). (C) The content of CAT, H₂O₂, T-SOD and GSH-Px in piglet's plasma (n=12/group). T-AOC: Total Antioxidant Capacity, CAT: Catalase, H₂O₂: Hydrogen Peroxide, MDA: Malondialdehyde, T-SOD: Total Superoxide Dismutase, GSH-Px: Glutathione Peroxidase.

NMN inhibits placental oxidative stress-induced mitophagy and promotes placental angiogenesis

The measurement of antioxidant-related gene expression in the placenta showed that supplementing the gestational diet with NMN significantly increased the mRNA expression levels of *SOD2* and *ATF4*, as well as the protein expression level of HO-1 (Figures 3G & K & P & R). HE staining demonstrated that the addition of 2 g/d NMN to the pregnancy diet significantly enhanced placental vascular density compared to the NC group. Correlation analysis revealed a significant positive association between placental vascular density and piglet birth weight (Figures 3A-C). Furthermore, the addition of 2 g/d NMN to the pregnancy diet significantly increased the mRNA expression levels of angiogenesis-related genes *CD31* and *VEGFR2* in the placenta. Adding 5 g/d NMN to the pregnancy diet significantly increased SIRT1 expression in the placenta, along with the mRNA expression levels of angiogenesis-related genes *CD31* and *VEGF-A*, and the protein expression level of Ang1 (Figures 3D-F & M & O & R). Immunofluores-

cence (IF) staining further indicated that the addition of 2 g/d NMN to the gestational diet significantly increased the fluorescence intensity of the angiogenesis marker CD31 in the placenta (Figures 3Q-S). These findings suggest that adding NMN to pregnancy diets alleviates placental oxidative stress, increases SIRT1 expression, and promotes placental angiogenesis.

The measurement of mitophagy-related genes in the placenta revealed that the addition of 2 g/d NMN to the gestational diet reduced the mRNA expression levels of *LC3B/LC3A* and the protein expression levels of Atg5 compared to the NC group. Similarly, the addition of 5 g/d NMN in the gestational diet decreased both the mRNA and protein expression levels of Atg5 (Figures 4A-B). The addition of NMN to the gestational diet significantly increased the mRNA and protein expression levels of p62 in the placenta (Figures 4C & G & L). Immunofluorescence staining indicated that NMN supplementation significantly decreased the fluorescence intensity of Parkin in the placenta (Figures 4J-K). These findings suggest that the addition of NMN to the gestational diet inhibits mitophagy in the placenta.

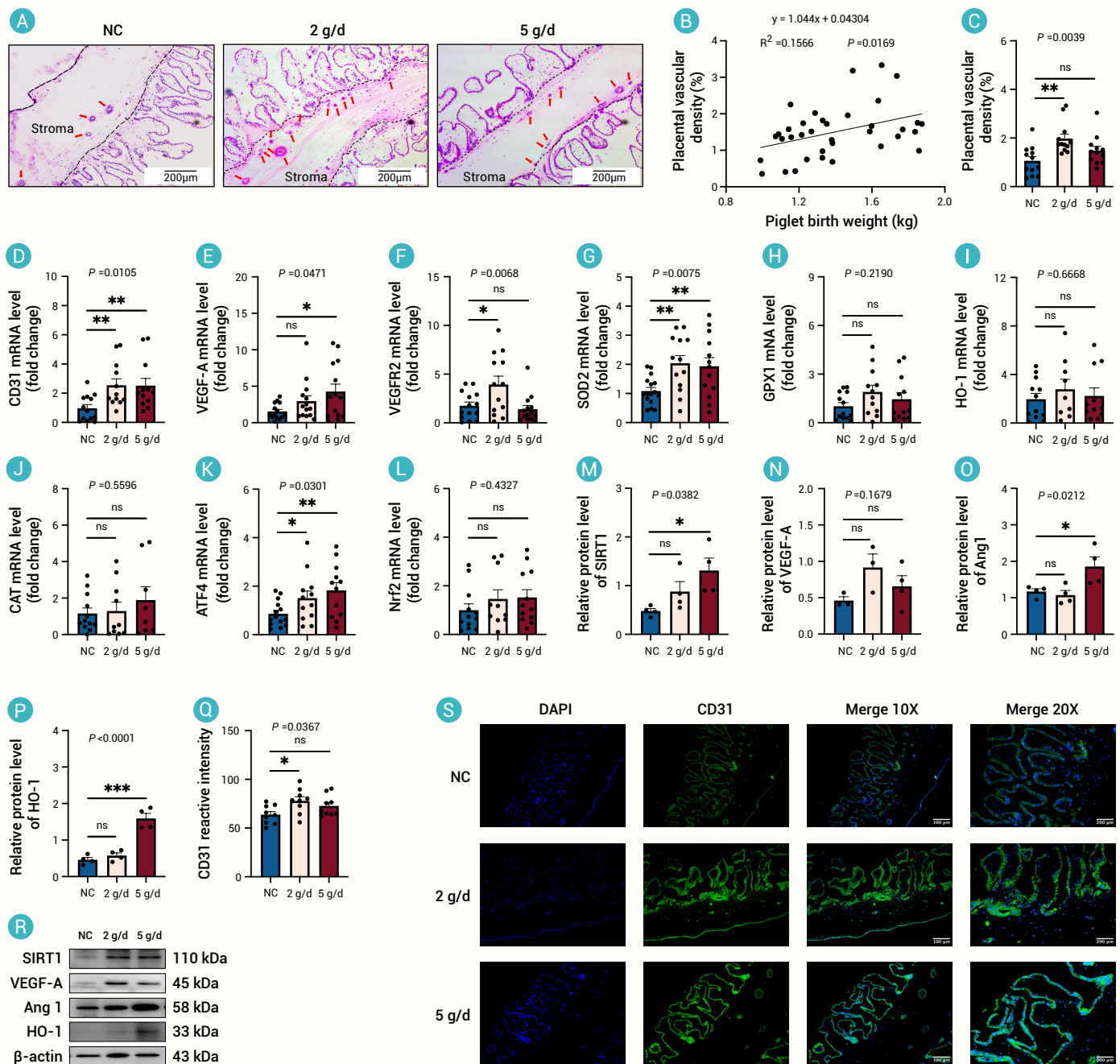


Figure 3. Effects of adding NMN to pregnancy diets on placental angiogenesis and oxidative stress status (A) Placental HE staining, with red arrows indicating placental blood vessels. Scale bar=200 μm (n=12/group). (B-C) Analysis of placental vascular density and its correlation with the birth weight of piglets. (D-L) The effect of adding NMN to the gestational diet on the mRNA expression of angiogenesis and antioxidant-related genes in the placenta (n=12/group). (M-P, R) The effect of adding NMN to the gestational diet on the protein expression of angiogenesis and antioxidant-related genes in the placenta (n=4/group). (Q, S) Placental IF staining. Scale bar=200 μm (n=9/group).

Oxidative stress model confirms that NMN promotes angiogenesis by inhibiting excessive mitophagy

An oxidative stress model was established in porcine vascular endothelial cells (PVECs) using H_2O_2 to investigate the effects of NMN on angiogenesis under oxidative stress conditions (The results of the CCK8 test showed that the optimal drug concentration of SIRT1720, NMN and H_2O_2 were 10 μM, 150 μM and 800 μM, Figures S2A-B & E). The results demonstrated that H_2O_2 treatment significantly reduced the mRNA expression levels of antioxidant-related genes, including *Nrf2*, *HO-1*, *GPX1*, *SOD2*, and *CAT* in PVECs. However, application of the SIRT1 activators NMN or SIRT1720 partially restored the expression levels of these genes, confirming the successful establishment of the oxidative stress model in PVECs (Figures 5J-N). In comparison to the NC group, oxidative stress reduced the migration and tube-

forming abilities of PVECs, whereas treatment with the SIRT1 activators NMN or SIRT1720 restored these functions (Figures 5A-F). Additionally, oxidative stress significantly decreased the mRNA levels of angiogenesis-related genes, including *VEGF-A*, *VEGFR2*, and *PLGF*, as well as the protein expression of VEGF-A in PVECs, while increasing overall acetylation levels in the placenta. NMN or SIRT1720 treatment partially reversed these changes, restoring angiogenesis-related gene expression (Figures 5G-I & P-Q & T-U).

When mitophagy occurs, aged or damaged mitochondria are enveloped by autophagic vesicles and fused with lysosomes.⁵⁵ To further investigate the mitophagy in PVECs, a mitochondrial-lysosome fluorescence probe double-staining assay was performed. The results indicated that oxidative stress or H_2O_2 treatment increased the fluorescence intensity of Parkin in PVECs compared to the NC group, while the addition of NMN decreased the fluores-

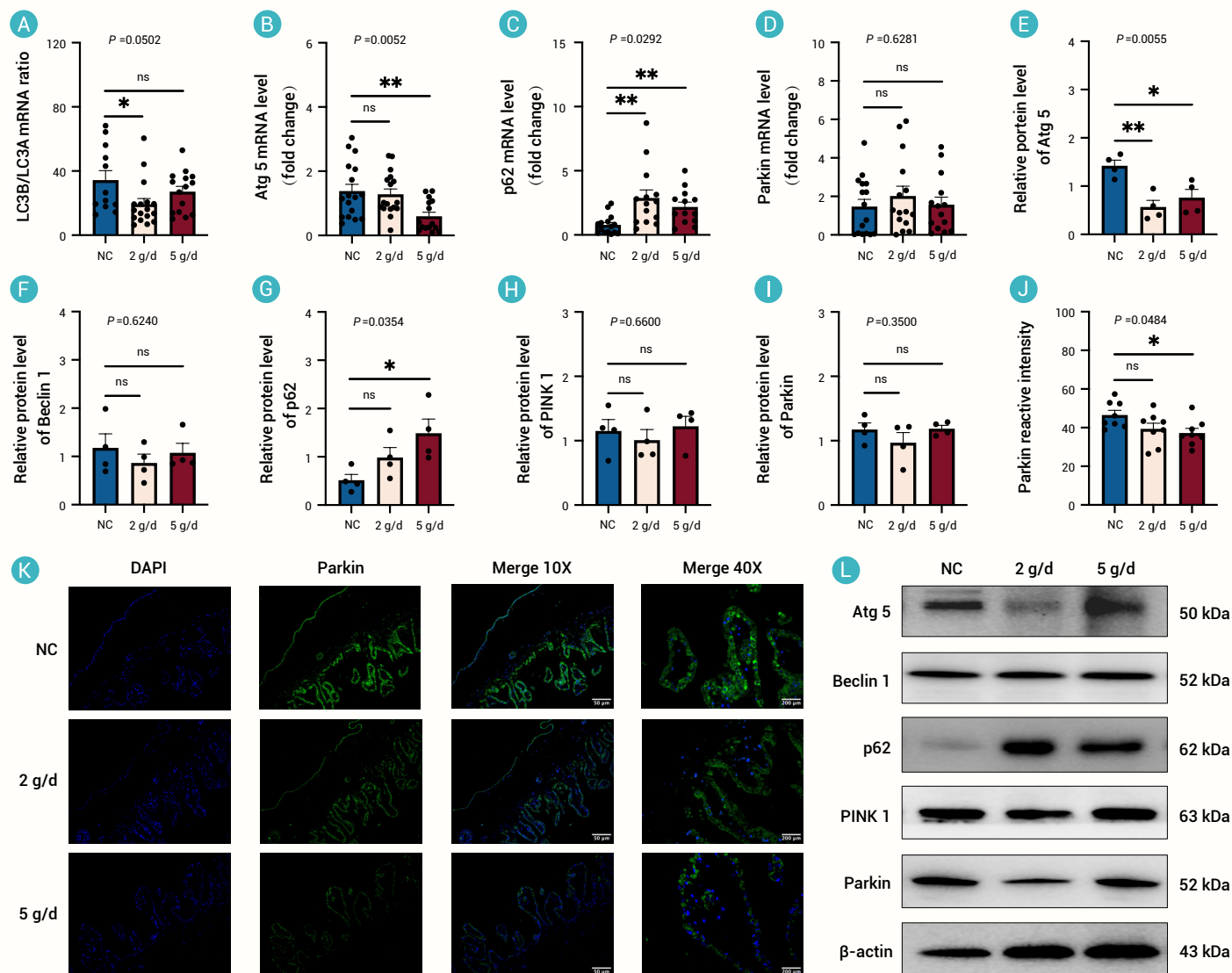


Figure 4. The effect of adding NMN to pregnancy diet on placental mitophagy (A-D) The effect of adding NMN to the pregnancy diet on the mRNA expression of mitophagy related genes in the placenta (n=12/group). (E-I, L) The effect of adding NMN to the pregnancy diet on the expression levels of mitophagy related gene proteins in the placenta (n=4/group). (J, K) Placental immunofluorescence staining, scale bar = 200 μm (n=9/group).

cence intensity (Figures 6A-B). Oxidative stress elevated the mRNA ratio of the mitophagy-related genes *LC3B/LC3A* and increased Beclin1 protein expression in PVECs, while decreasing the mRNA and protein expression of p62 (Figures 6C & F-G & J). These findings demonstrate that NMN can mitigate excessive mitophagy in PVECs activated by oxidative stress. In addition, mitochondrial-lysosome fluorescence probe double staining indicated that oxidative stress significantly increased the degree of mitochondrial-lysosome fluorescence co-localization in PVECs compared to the NC group, while NMN decreased the degree of co-localization (Figures 6K-L). These findings suggest that oxidative stress induces excessive mitophagy, and NMN alleviates oxidative stress, inhibits excessive mitophagy, and promotes angiogenesis in PVECs.

NMN-SIRT1 is a positive regulatory factor for mitophagy and angiogenesis in PVECs

To further elucidate the relationship between NMN-SIRT1 signaling, mitophagy, and angiogenesis, we treated non-oxidative stressed PVECs with the SIRT1 activator SRT1720 and the inhibitor EX527 (The results of the CCK8 test showed that the optimal drug concentration of EX527 was 15 μM, Figure S2C). This approach aimed to investigate the role of NMN-SIRT1 signaling in mitophagy and angiogenesis in PVECs. Scratch healing experiments demonstrated that treatment with NMN or SRT1720 significantly

increased the migratory ability of PVECs compared to the NC group, while EX527 treatment reduced the migratory capacity of PVECs (Figures 7A-B). Furthermore, NMN-SIRT1 effect on tube formation was evaluated in an in vitro Matrigel experiment. Activation of NMN-SIRT1 significantly increased both the number of nodes and the length of branches in PVECs. However, inhibition of NMN-SIRT1 had no significant effect on the tube-forming ability of PVECs (Figures 7C & E-G). Additionally, activation of NMN-SIRT1 significantly increased the mRNA expression levels of *CD31* and *PLGF*, as well as the protein expression of VEGF-A in PVECs, compared to the NC group. Conversely, inhibition of NMN-SIRT1 decreased the protein expression level of Ang 1 in PVECs (Figures 7D & H & K & P-Q). These results suggest that NMN-SIRT1 is a key target for promoting angiogenesis.

We also examined the expression of mitophagy-related genes and proteins in PVECs. Activation of NMN-SIRT1 significantly increased the mRNA expression levels of *LC3B/LC3A* and *Parkin*, as well as the protein expression levels of ATG5, Beclin1, PINK1, and Parkin in PVECs (Figures 7L & N). In contrast, inhibition of NMN-SIRT1 decreased the protein expression level of Beclin1 in PVECs and increased the protein expression level of p62 (Figures 7D & S-T). Furthermore, activation of NMN-SIRT1 increased the fluorescence co-localization of mitochondria and lysosomes in PVECs compared to the NC group, while inhibition of NMN-SIRT1 expression decreased their fluorescence co-localization (Figures 7W-X). These results

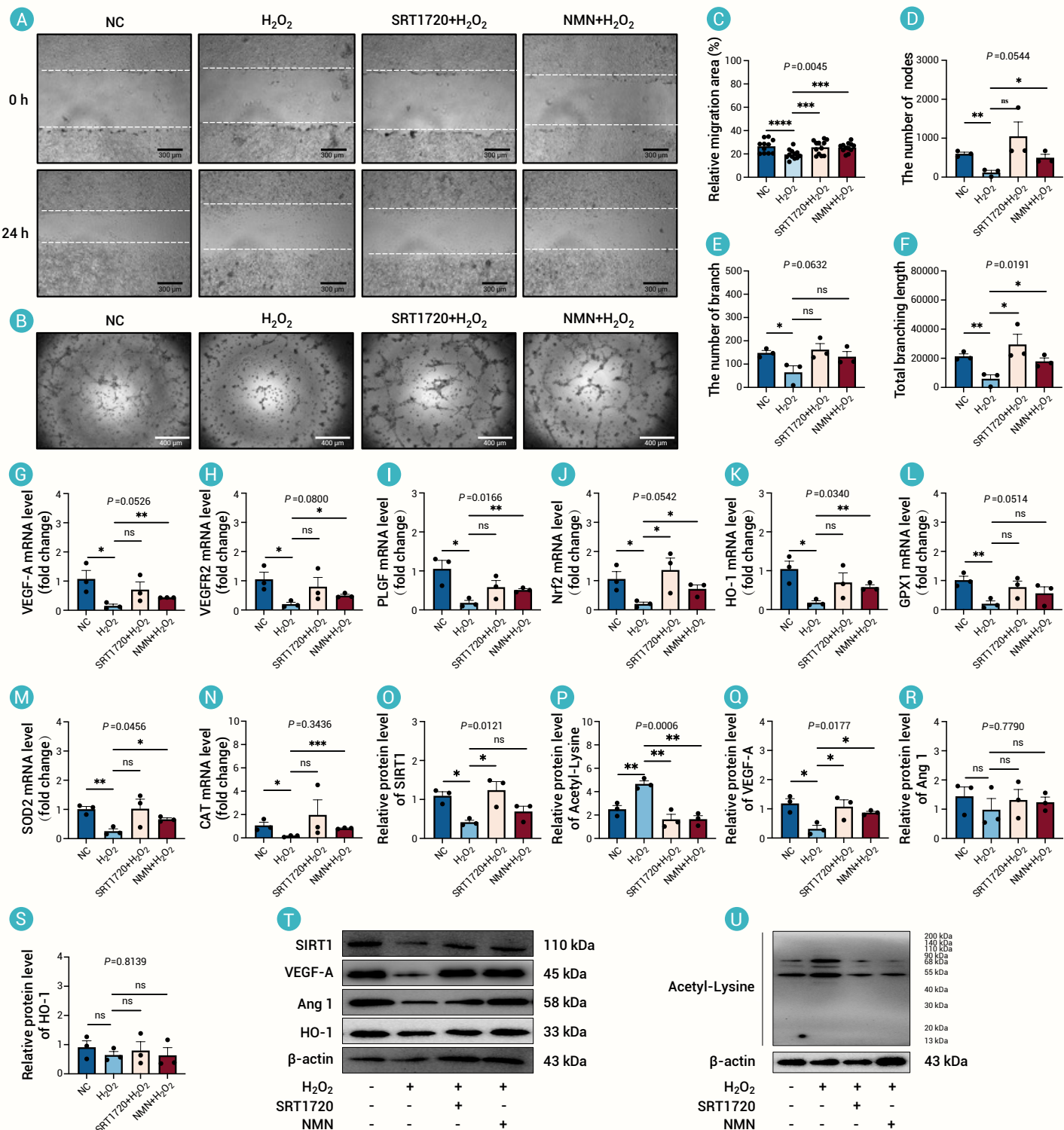


Figure 5. Effects of adding NMN on angiogenesis and oxidative stress status of PVECs under oxidative stress (A, C) Effects of a 24-hour treatment with H_2O_2 , H_2O_2 + SRT1720, and H_2O_2 + NMN on the migration ability of PVECs. Scale bar = 300 μm ($n=12$ /group). (B, D-F) Effects of a 24-hour treatment with H_2O_2 , H_2O_2 + SRT1720, and H_2O_2 + NMN on the tube formation ability of PVECs. Scale bar = 400 μm ($n=3$ /group). (G-U) Effects of a 24-hour treatment with H_2O_2 , H_2O_2 + SRT1720, and H_2O_2 + NMN on the mRNA expression and protein levels of total acetylation, angiogenesis, and antioxidant-related genes in PVECs ($n=3$ /group).

suggest that NMN-SIRT1 promotes mitophagy and angiogenesis in PVECs.

Angiogenesis promoted by NMN-SIRT1 in PVECs is dependent on mitophagy

To further clarify the relationship between NMN-SIRT1 signaling and mitophagy under non-oxidative stress conditions, we treated PVECs with the mitophagy inhibitor 3-MA (The results of the CCK8 test showed that the optimal drug concentration of 3-MA was 2 mM, Figure S2D). Additionally, NMN

and the SIRT1 activator SRT1720 were administered to observe the effects of NMN-SIRT1 activation on the angiogenesis in PVECs when mitophagy was inhibited. The results showed that treatment with 3-MA significantly reduced the mRNA expression levels of mitophagy-related genes, including *ATG5* and *Parkin*, as well as the protein expression levels of Beclin1, PINK1, and Parkin in PVECs. However, activation of NMN-SIRT1 expression partially restored the expression of these mitophagy-related genes in PVECs (Figures 8D & M-N & S & U-V). Mitochondrial-lysosome fluorescence probe double staining

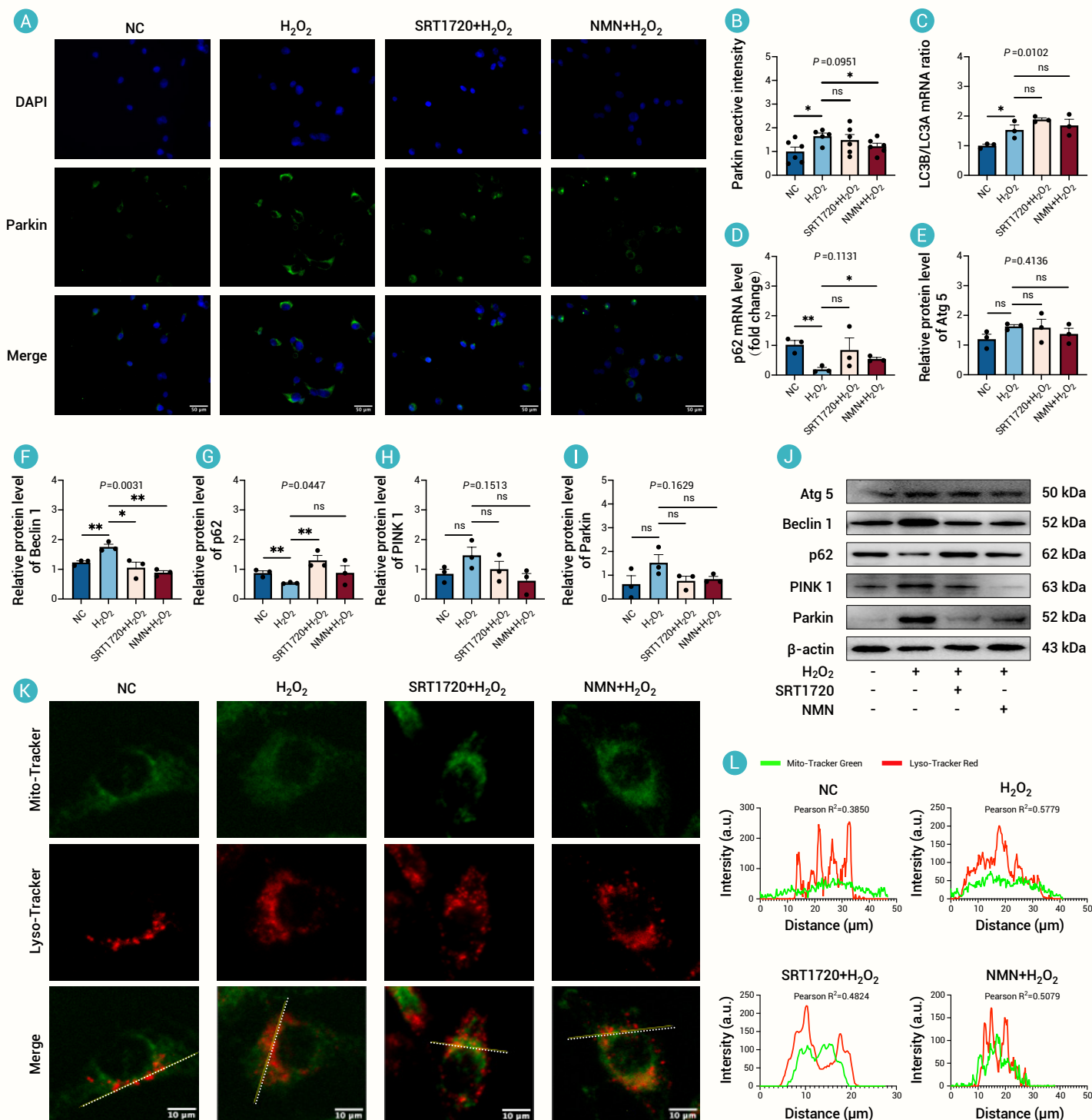


Figure 6. The effect of adding NMN on mitophagy in PVECs under oxidative stress (A, B) Immunofluorescence staining of cells. Scale bar = 50 μ m (n=6/group). (C-J) Effects of a 24-hour treatment with H₂O₂, H₂O₂ + SRT1720, and H₂O₂ + NMN on the mRNA expression and protein levels of mitochondria-autophagy related genes in PVECs (n=3/group). (K, L) Effects of a 24-hour treatment with H₂O₂, H₂O₂ + SRT1720, and H₂O₂ + NMN on the degree of mitochondrial-lysosome fluorescence colocalization in PVECs. Scale bar = 10 μ m (n=3/group).

indicated that 3-MA treatment decreased the co-localization of mitochondria and lysosomes in PVECs compared to the NC group. However, NMN-SIRT1 activation restored this co-localization (Figures 8W-X), suggesting that NMN-SIRT1 mediates mitophagy in PVECs. Inhibition of mitophagy in PVECs significantly decreased their ability to migrate and form tubes. However, activation of NMN-SIRT1 expression partially restored the migration and tube formation capabilities of PVECs (Figures 8A-C & E-G). Treatment with 3-MA significantly decreased the mRNA expression levels of *CD31*, *VEGF-A*, *VEGFR2*, and *PLGF*, as well as the protein expression levels of VEGF-A in

PVECs compared to the NC group. Activation of NMN-SIRT1 restored these expression levels (Figures 8D & H-K & P). These results confirm that NMN-SIRT1 partially promotes angiogenesis by activating mitophagy.

DISCUSSIONS

Oxidative stress is a critical factor contributing to adverse pregnancy outcomes.²⁶ The placenta, a temporary organ formed during pregnancy, plays a key role in supporting fetal development by secreting hormones, growth factors, and cytokines.^{10-13,56} Oxidative stress-induced placental dysfunction

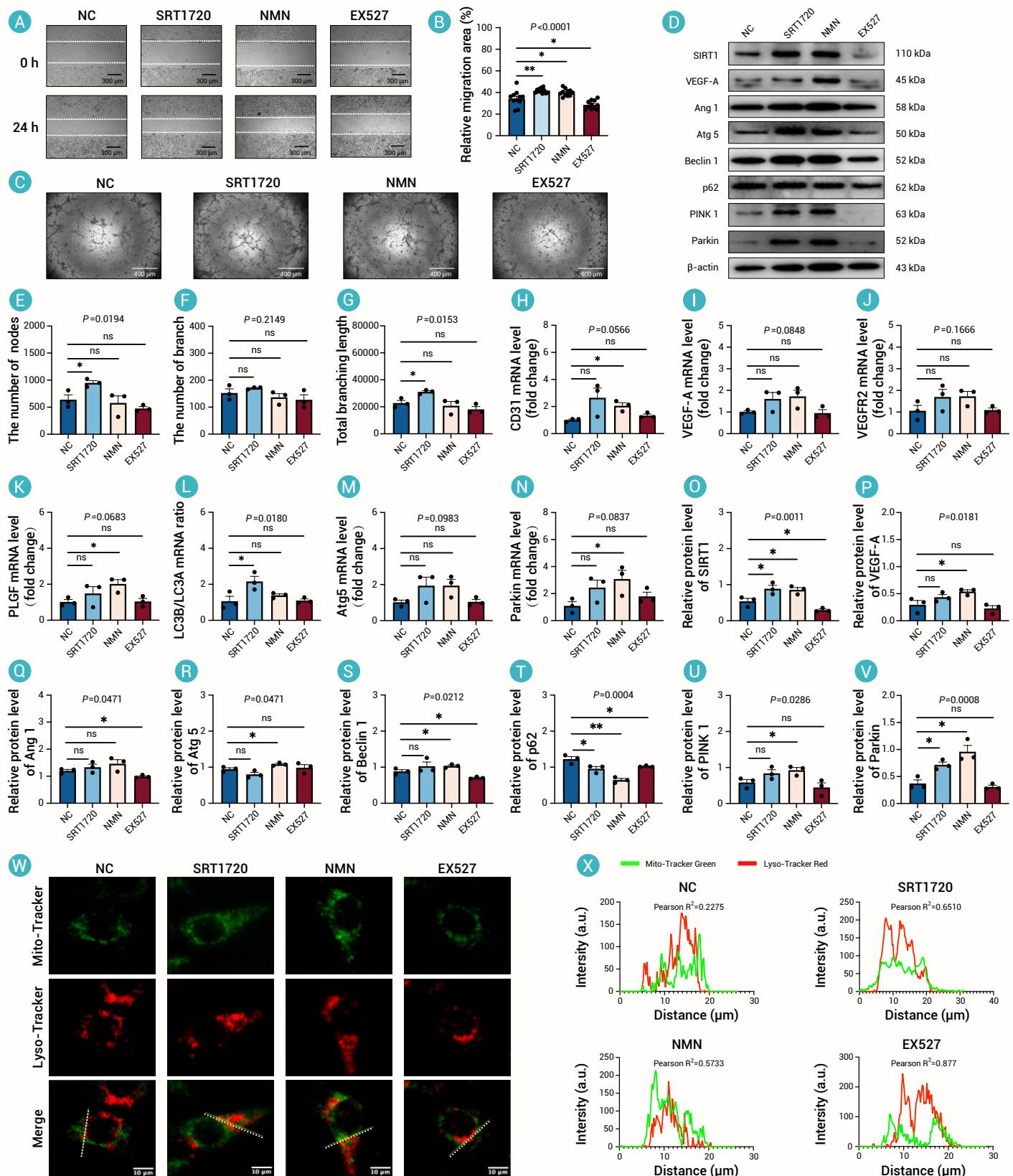


Figure 7. Effects of activation or inhibition of NMN-SIRT1 on angiogenesis and mitophagy in PVECs (A, B) Effects of a 24-hour treatment with SRT1720, NMN, and EX527 on the migratory ability of PVECs. Scale bar = 300 μ m (n=12/group). (C, E-G) Effects of a 24-hour treatment with SRT1720, NMN, and EX527 on the tube formation ability of PVECs. Scale bar = 400 μ m (n=3/group). (D, H-V) Effects of a 24-hour treatment with SRT1720, NMN, and EX527 on the mRNA expression and protein levels of angiogenesis and mitochondria-autophagy related genes in PVECs (n=3/group). (W, X) Effects of a 24-hour treatment with SRT1720, NMN, and EX527 on the degree of mitochondrial-lysosome fluorescence colocalization in PVECs. Scale bar = 10 μ m (n=3/group).

can result in delayed villus maturation, insufficient fetal vascular perfusion, IUGR, preterm birth, and stillbirth, among other complications.⁵⁷ In sow

production, research has demonstrated that IUGR piglets exhibit reduced placental vascular density and elevated oxidative stress levels.¹⁵⁻¹⁷ In vitro

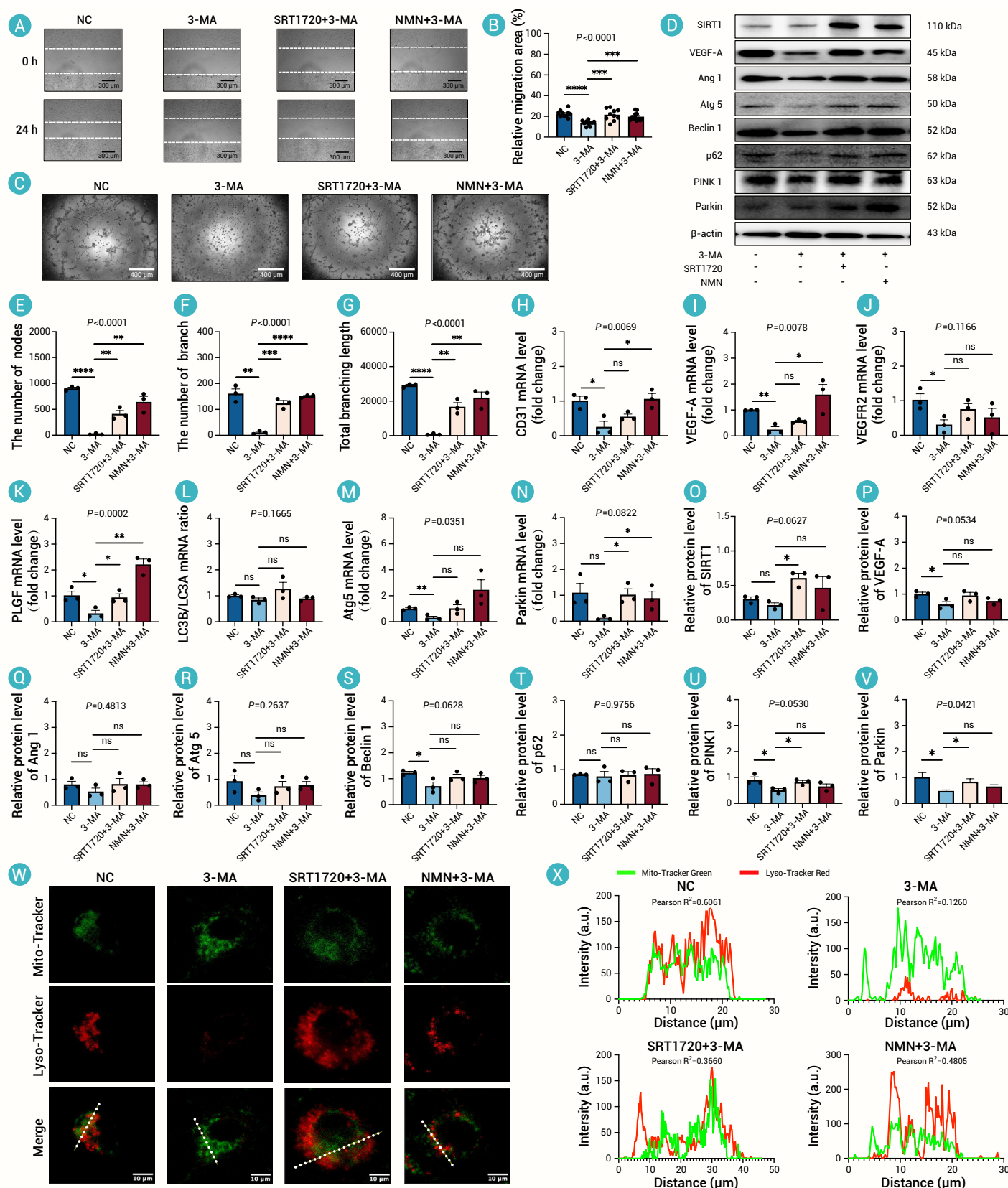


Figure 8. Effects of activating NMN-SIRT1 after inhibiting mitophagy on angiogenesis and mitophagy in PVECs. (A, B) Effects of a 24-hour treatment with 3-MA, 3-MA + SRT1720, and 3-MA + NMN on the migration ability of PVECs. Scale bar = 300 μ m (n=12/group). (C, E-G) Effects of a 24-hour treatment with 3-MA, 3-MA + SRT1720, and 3-MA + NMN on the tube formation ability of PVECs. Scale bar = 400 μ m (n=3/group). (D, H-V) Effects of a 24-hour treatment with 3-MA, 3-MA + SRT1720, and 3-MA + NMN on the mRNA expression and protein levels of angiogenesis and mitophagy-related genes in PVECs (n=3/group). (W, X) Effects of a 24-hour treatment with 3-MA, 3-MA + SRT1720, and 3-MA + NMN on the degree of mitochondrial-lysosome fluorescence colocalization in PVECs. Scale bar = 10 μ m (n=3/group).

studies have also indicated that oxidative stress inhibits the proliferation and migration of human placental trophoblast cells (HTR8/SVneo), thereby

disrupting normal placental development.⁵⁸ Consequently, reducing ROS may be an effective strategy to mitigate placental dysfunction. Recent studies

have confirmed that supplementing pregnant sows with methionine, cysteine, allicin, and other compounds significantly alleviates maternal and placental oxidative stress levels, promotes placental angiogenesis and fetal development, and lowers the incidence of IUGR.²¹⁻²⁵ Here, pregnancy diet of sows with 2-5 g/d of NMN alleviated oxidative stress in sows, placentas, and their offspring, inhibited excessive mitophagy in the placenta, and promoted placental angiogenesis. Although the serum level of GSH-Px decreased in sows after NMN treatment in our study, we believe this may be because NMN significantly increased the activities of CAT and T-SOD in sows. This likely reduced the reliance on the GSH-Px pathway, which can be interpreted as a homeostatic response of sows following oxidative stress. Mechanistically, under perinatal oxidative stress, the NMN-SIRT1 signaling pathway promoted placental angiogenesis and fetal development. This occurred through the antioxidant effects of NMN and by inhibiting excessive mitophagy, which effectively reduced the incidence of IUGR in piglets. However, in the absence of oxidative stress, NMN-SIRT1 acts as a potent mitophagy-promoting signal, positively regulating PVEC angiogenesis.

Among various antioxidant compounds, NMN stands out due to its significant advantages. Naturally occurring in a wide range of plants and animals, NMN can be purified from bacteria and yeast. It is widely used in treating aging-related diseases such as type 2 diabetes, obesity, cerebral ischemia, heart ischemia, Alzheimer's disease, and other neurodegenerative disorders.^{39-42,59-60} The physiological functions of NMN are primarily mediated by NAD⁺, a crucial redox coenzyme in eukaryotic mitochondria. NAD⁺ plays a vital role in various biological processes, including cell death, aging, oxidative stress, inflammation, and DNA damage repair.⁶¹ However, research on the role of NMN in alleviating placental oxidative stress remains limited. Clinical studies in humans have demonstrated that single doses of NMN ranging from 1 to 500 mg/d are safe, with no observable effects on body temperature, oxygen saturation, blood pressure, or heart rate.^{42,62} Commercial NMN supplements typically contain doses ranging from 50 to 500 mg per capsule.⁴¹ Given that the body weight of multiparous sows typically ranges from 200 to 220 kg (approximately 2-3 times that of adult males), this experiment administered NMN at 2-5 g per sow per day. Remarkably, high doses of NMN did not induce any embryotoxicity. Instead, supplementation significantly increased the birth weight of piglets and reduced the proportion of low-birth-weight piglets. However, no further improvement in birth weight, litter weight, or the number of healthy piglets was observed when the dosage was increased from 2 g/d to 5 g/d, suggesting a threshold beyond which additional benefits are not realized. In addition, both *in vivo* and *in vitro* studies have demonstrated that NMN exhibits strong antioxidant activity, which may reduce oxidative stress in sows, placentas, and newborn piglets. Furthermore, NMN has been shown to enhance glucose and lipid metabolism in both mothers and their offspring. These findings indicate that NMN holds promising potential for alleviating oxidative stress during pregnancy, improving placental function by promoting angiogenesis, increasing placental and fetal weight, and reducing the incidence of IUGR.

In the process of angiogenesis, the proliferation, invasion, and migration of endothelial cells require a sufficient energy supply, and good mitochondrial function is essential for facilitating this process.^{20,36,63} However, mitochondria are also the primary source of ROS production in the body. Under oxidative stress, the persistent assault of ROS on mitochondria can result in structural and functional failure, triggering moderate mitophagy as a compensatory response.^{64,65} Mitophagy refers to the process by which the autophagy system targets damaged mitochondria and transports them to lysosomes for degradation, which is crucial for maintaining mitochondrial quality and quantity across various cell types.⁶⁶ In general, mitophagy is a consequence of ROS accumulation, aimed at reducing ROS levels and limiting prolonged exposure to oxidative stress.⁶⁷ However, excessive mitophagy may increase ROS production, adversely affecting normal cellular function, particularly when the organism is already in a compromised state. This excessive mitophagy can persist and even trigger programmed cell death.⁶⁸⁻⁷⁰ For example, in the case of Nephropathic Cystinosis, excessive mitochondrial autophagy results in abnormal mitochondrial function, reduced ATP production, and increased ROS production, ultimately promoting renal injury.⁷⁰ In melanoma cells, sustained mitophagy activation can elevate ROS levels, leading to necroptosis and ferroptosis.⁷¹ Zhou et al.⁷² found that in flap-trans-

planted rats, flap ischemia-reperfusion injury (I/R) increased ROS production, activated PINK1/Parkin-mediated mitophagy, and subsequently suppressed VEGF expression, inhibited angiogenesis, and reduced the microvessel density at the flap grafting site. In this study, sows in the NC group exhibited higher levels of oxidative stress and mitophagy, while the expression of genes related to placental angiogenesis was inhibited. This finding aligns with results from H₂O₂-induced excessive mitophagy in PVECs, which restricted angiogenesis. These results suggest that oxidative stress damage in perinatal sow organisms activated excessive mitophagy in the placenta, inhibiting placental angiogenesis and fetal development. However, the addition of NMN alleviated oxidative stress-induced mitophagy, thereby protecting the placental endothelium and facilitating nutrient transport. A recent study found that perinatal exposure to glyphosate-based herbicides increased oxidative stress in sows, impaired mitochondrial function in the placenta, and led to vascular limitations.⁷³ Research in humans also showed elevated mitochondrial ROS levels in both preeclampsia and IUGR placentas, along with dysfunctional mitochondria and reduced placental blood perfusion.^{47,48} In a non-oxidative stress PVEC model, NMN was shown to promote mitophagy, which in turn enhanced angiogenesis. This finding supports previous research indicating that NMN facilitated mitophagy in other physiological contexts. For example, Lu et al.⁷⁴ demonstrated that NMN activated mitophagy by downregulating p62 expression and upregulating LC3B expression, alleviating TNF- α -induced senescence and differentiation inhibition in human primary osteoblasts (HOB) and promoting bone healing in osteoporotic mice. Forte et al.⁷⁵ showed that intraperitoneal injection of NMN at a dose of 250 mg/kg/d in hypertensive rats significantly activated mitophagy in the brain, mitigating hypertension-induced cerebrovascular injury. Although the results confirm that NMN holds significant potential to improve placental function, further clinical trials are necessary to validate its safety and efficacy during pregnancy. This will enhance the application value of NMN in pregnancy health management.

It is widely accepted that NMN, as a precursor to NAD⁺ synthesis, primarily regulates mitochondrial homeostasis through SIRT1, thereby improving oxidative stress levels in tissues and organs.^{37,39,43,76} When the body's NAD⁺ content decreases, SIRT1 influences mitochondrial biogenesis by increasing PGC1 α acetylation levels and simultaneously disrupts communication between mitochondria and the nucleus through the SIRT1-HIF-1 α -OXPHOS pathway. This disruption leads to mitochondrial imbalance, contributing to age-related complications and disease development.^{44,77} These findings align with the observed increase in SIRT1 protein levels, enhanced deacetylase activity, and reduced oxidative stress levels following NMN supplementation in this study. It has been reported that the activation of NMN-SIRT1 in aging rat models promotes the regeneration of cerebral endothelium and restores blood flow and cognitive function by enhancing the proliferation and migration abilities of brain microvascular endothelial cells (CMVEC).⁷⁸ These results indicate that the NMN-SIRT1 signaling pathway plays a critical role in improving endothelial function. However, the mechanisms by which SIRT1 influences endothelial mitophagy under non-oxidative stress, as well as the relationship between mitophagy and angiogenesis, remain unclear. In this study, we found that NMN-SIRT1 signaling activated mitophagy and promoted angiogenesis in PVECs under non-oxidative stress conditions. Although treatment with the mitophagy inhibitor 3-MA significantly inhibited both mitophagy and angiogenesis, NMN and SIRT1720 treatment still alleviated the 3-MA-induced inhibition of mitophagy and promoted angiogenesis. These results suggest that NMN-SIRT1 signaling acts as a positive regulator of mitophagy and angiogenesis in PVECs under non-oxidative stress injury, indicating that NMN-SIRT1 can enhance angiogenesis by activating mitophagy. Although this study indicates that the activation of the NMN-SIRT1 signaling pathway promoted placental angiogenesis and fetal development by modulating mitophagy flux in both oxidative and non-oxidative stress states, the detailed mechanism of the interaction between SIRT1 and mitophagy remains to be elucidated.

CONCLUSIONS

In summary, the present study demonstrates that under perinatal oxidative stress, the NMN-SIRT1 signaling pathway promoted placental angiogenesis and fetal development. This occurred through the antioxidant effects of NMN and by inhibiting excessive mitophagy, which effectively reduced the

incidence of IUGR in piglets. Additionally, NMN-SIRT1 signaling enhanced placental angiogenesis by activating mitophagy under non-oxidative stress conditions. In conclusion, this research uncovers how NMN regulates placental angiogenesis in sows and provides new nutritional regulatory targets to reduce the incidence of IUGR in piglets even in humans.

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AUTHOR CONTRIBUTIONS

J.P., Y.Z.: conceptualization, investigation, methodology, formal analysis, data curation, writing-original draft, writing-review & editing, visualization; K.G.: investigation, methodology, formal analysis, data curation; S.Z., W.Z., Z.L., Y.H.: methodology, formal analysis, investigation, data curation; K.Y., Y.H., W.W., J.W., C.T., D.W.: methodology, writing-review & editing; R.L., Y.Y., Y.Z.: project administration, supervision, writing-review & editing. All the authors read and agreed to publish the final version of the manuscript.

DECLARATION OF INTERESTS

Yulong Yin is an Editorial Board member of *The Innovation Life*. He was blinded from reviewing or making final decisions on the manuscript. The article was subject to the journal's standard procedures, with peer review handled independently of these Editorial Board members and their research groups. The other authors declare that they have no conflicts of interest.

ETHICAL STATEMENT AND PATIENT CONSENT

The authors declare that they have no conflict of interest. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee of Zhongkai University of Agriculture and Engineering (Ethical approval number, ZHKU-2023-0220).

DATA AND CODE AVAILABILITY

All data are available in the main text or the supplementary materials.