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Original Research Article

# Dietary adenosine supplementation improves placental angiogenesis in IUGR piglets by up-regulating adenosine A2a receptor

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ABSTRACT

Abnormal placental angiogenesis is associated with the occurrence of intrauterine growth restriction (IUGR) in piglets, and effective treatment strategies against this occurrence remain to be explored. Adenosine has been reported to play an important role in angiogenesis, but its role in placental angiogenesis is still unknown. Here, we investigated the effect of dietary adenosine supplementation on IUGR occurrence in piglets by analyzing the role of adenosine in placental angiogenesis for Normal and IUGR piglets. Specifically, 88 sows were allotted to 2 treatments ($n = 44$) and fed a basal diet supplemented with 0% or 0.1% of adenosine from day 65 of gestation until farrowing, followed by collecting the placental samples of Normal and IUGR piglets, and recording their characteristics. The results showed that adenosine supplementation increased the mean birth weight of piglets ($P < 0.05$) and placental efficiency ($P < 0.05$), while decreasing the IUGR piglet rate ($P < 0.05$). Expectedly, the placenta for IUGR neonates showed a down-regulated vascular density ($P < 0.05$) and angiogenesis as evidenced by the expression level of vascular cell adhesion molecule-1 (VCAM1) ($P < 0.05$). Notably, dietary adenosine supplementation promoted angiogenesis ($P < 0.05$) both in the Normal and IUGR placenta. More importantly, the expression level of adenosine A2a receptor (ADORA2A) was lower ($P < 0.05$) in the IUGR placenta than in Normal placenta, whereas adenosine treatment could significantly increase ADORA2A expression, and also had an interaction effect between factors IUGR and Ado. Collectively, placentae for IUGR piglets showed impaired angiogenesis and down-regulated expression level of ADORA2A, while dietary adenosine supplementation could activate ADORA2A expression, improve the placental angiogenesis, and ultimately decrease the occurrence of IUGR in piglets.

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## 1. Introduction

As a complication of pregnancy, intrauterine growth restriction (IUGR) can cause a low birth weight (LBW) for neonates, and about 15% to 25% of piglets were reported to suffer from IUGR in pig production (Freking et al., 2016). IUGR can bring a series of adverse consequences to piglets, such as a higher rate of morbidity and mortality, slower growth rate, as well as abnormal organ development (Oksbjerg et al., 2013; Wu et al., 2006), suggesting a beneficial effect of reducing IUGR rate on the reproductive efficiency of sows. However, the mechanism underlying IUGR occurrence is still largely unclear.

It is essential for the placenta to efficiently transfer oxygen and nutrients from the mother to the fetus to maintain normal fetal

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growth, and the blood vessels in the placenta play an important role in the exchange of maternal–fetal material (Reynolds et al., 2006; Sun et al., 2020; Zhang et al., 2022). Previous studies revealed abnormal angiogenesis in LBW placenta (Hu et al., 2021; Huang et al., 2021a), implying that improving angiogenesis in the placenta might decrease IUGR occurrence. As an endogenous purine nucleoside, adenosine exerts its biological effects by activating its 4 adenosine receptor subtypes, including adenosine A1 receptor (ADORA1); adenosine A2a receptor (ADORA2A); adenosine A2b receptor (ADORA2B) and adenosine A3 receptor (ADORA3) (Salsoso et al., 2017). Previous studies have confirmed the promoting role of adenosine in biological and pathological angiogenesis (Antonioli et al., 2021; Troncoso et al., 2020; Valls et al., 2021). Additionally, adenosine was reported to activate ADORA2A to potentiate angiogenesis by regulating the levels of angiogenesis factors including vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1$\alpha$ (HIF-1$\alpha$) (Escudero et al., 2013; Fernandez et al., 2012; Liu et al., 2017). However, the effect of adenosine on placental angiogenesis remains to be further investigated.

In this study, adenosine was assumed to alleviate IUGR occurrence by enhancing placental angiogenesis, and this hypothesis was tested by analyzing the effects of adenosine on the occurrence of IUGR piglets and comparing the angiogenesis and the expression of adenosine receptors in placentae for Normal and IUGR piglets.

## 2. Materials and methods

### 2.1. Animal ethics statement

All animal experimental design and procedures presented in this study were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Science, and performed according to the Guidelines for Care and Use of Laboratory Animals of South China Agriculture University (Guangzhou, China).

### 2.2. Animals and experimental design

The sows used in this study were obtained from Jiangxi Wannian Xinxing Agro-pastoral Co., Ltd., China. A total of 88 Duroc × Landrace × Yorkshire sows were divided into 2 dietary treatment groups, each sow as a replicate of a completely randomized design, based on the body weight at day 65 of gestation ($n = 44$ per treatment). From day 65 of gestation to farrowing, the control group (Con) sows were fed a basal gestation diet without adenosine supplementation, while the adenosine group (Ado) sows were fed a basal diet with 0.1% adenosine supplementation. Adenosine was from Hangzhou Kaipeng Biotechnology Co., Ltd., Hangzhou, China, purity (HPLC) ≥ 98.0%. Both diets were formulated to meet the National Research Council (NRC, 2012) nutritional standards for gestational sows. The composition of the diet is shown in Supplementary Table S1. The feed intake of the sows is shown in Supplementary Table S2.

### 2.3. Data collection and sampling

After farrowing, the number and weight of piglets born were recorded. The IUGR rate was calculated as previously described (Aditya et al., 2016). Briefly, IUGR piglets were defined as piglets with a birth weight of 2 standard deviations lower than the mean birth weight of the Con group piglets. When piglets were born, the umbilical cord was immediately tied with a silk line (labeled with a number tag to indicate the birth order of the piglet), and each piglet was marked with a numbered tag to match the placenta. The newborn piglet birth weight and numerical order were recorded, the placenta weight was also recorded after placental expulsion. Then, approximately 10 g of the placenta (3 to 4 cm from the cord insertion point) was collected and snap-frozen immediately in liquid nitrogen, and another fresh placental tissue was collected and fixed in 4% paraformaldehyde immediately. The placental efficiency was calculated by dividing piglet weight by placental weight (Wilson et al., 1999). The average birth weight of 441 piglets in the Con group in this study was 1.37 ± 0.15 kg (mean ± SD), and the placental samples were divided into 2 groups according to piglet birth weight: <1.07 kg (IUGR) and 1.37 to 1.52 kg (Normal). Six sows were randomly selected from each group, and each sow was randomized to provide 1 placenta of Normal fetal (piglet with birth weight 1.37 to 1.52 kg) and 1 placenta of IUGR fetal (piglet with birth weight <1.07 kg) for following analysis.

### 2.4. Placental vascular density

Six sows were selected from each group, and 2 placental samples of each sow were analyzed, including 1 Normal and 1 IUGR placental sample. By image analysis, the number of blood vessels was determined by estimating the mean value of 3 slices of 1 placenta. Briefly, fresh placental tissues fixed in 4% paraformaldehyde were paraffin-embedded and sectioned at 5 μm thickness, then stained with hematoxylin and eosin (H&E). The areas occupied by placental tissues and the placental vessels in these areas were traced with a projecting microscope (Olympus CX41, Japan). For each of the 5 μm sections, the total number of vessels in the placental stromal areas were determined and then corrected for the measured total placental stromal areas (per unit area as mm$^2$).

### 2.5. Quantitative real-time RT-PCR analysis

Total RNA from placental tissues was extracted with the reagent box of a Tissue RNA Purification Kit (EZBioscience, Suzhou, China) as instructed by the manufacturer. The concentration of RNA was quantified using a NanoDrop-2000 (Thermo Fisher, USA). After reverse transcription using a Color Reverse Transcription Kit (EZBioscience, Suzhou, China), qRT-PCR was conducted using SYBR Green on a QuantStudio 6 RealTime PCR System (Thermo Fisher, USA) under the conditions of denaturation at 95 °C for 10 min, amplification at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Each target gene was individually normalized to the reference gene 18S rRNA by using the quantification method of $2^{-\Delta\Delta ct}$. Primers used in this study are shown in Supplemental Table S3.

### 2.6. Western blotting

Total proteins were extracted from placental tissues using a protein extraction kit (Beyotime, Beijing, China) as informed by the manufacturer. Briefly, 10 μg of protein was loaded and separated by SDS-PAGE gel electrophoresis, and then the protein was transferred onto a polyvinylidene difluoride membrane (Merck Millipore). After blocking with TBST buffer containing 5% milk, the blots were then incubated overnight at 4 °C with each of the following primary antibodies: angiogenin (Ang) (ab95389, abcam, 1:1000), ADORA1 (ab82477, abcam, 1:1000), ADORA2A (ab3461, abcam, 1:1000), ADORA2B (ab222901, abcam, 1:1000), ADORA3 (ab197350, abcam, 1:1000), vascular endothelial growth factor A (VEGF-A) (19003-1-AP, Proteintech, USA, 1:1000), Akt (9272, CST, 1:1000), p-Akt (4060, CST, 1:1000), signal transducer and activator of transcription-3 (Stat3) (ab76315, Abcam, USA, 1:1500), p-Stat3 (ab68153, Abcam, USA, 1:1500), vascular cell adhesion molecule-1 (VCAM1) (ab134047, abcam, 1:1000), and β-actin (4970, CST, USA, 1:1000). Subsequently, the membranes were incubated with appropriate HRP-conjugated anti-rabbit IgG secondary antibody

(AS014, Abclonal, China, 1:5000). Images were captured using the ChemiDoc MP system (Bio-Rad, Hercules, CA, USA), and band densities were quantified using Image Lab soft-ware (Bio-Rad, Hercules, CA, USA) and then normalized to β-actin content.

### 2.7. Immunofluorescence

Placental tissues immobilized in 4% paraformaldehyde were embedded in paraffin and sectioned at 5 μm thickness for platelet endothelial cell adhesion molecule-1 (CD31) and ADORA2A immunofluorescence. Slides were visualized under a fluorescent microscope (Nikon Eclipse C1, Tokyo, Japan) and quantified by ImageJ software.

### 2.8. Statistical analysis

All data were presented with bar charts using GraphPad Prism (GraphPad Software, La Jolla, CA) and each bar represents the mean ± standard error of the mean (SEM). For sow and litter data, the sow or the litter represented the experimental unit. Statistical significance of reproductive performance (piglet mean BW at birth and placental efficiency) was determined by unpaired Student's *t*-test using SPSS 20.0 (SPSS Inc., Chicago, USA) software. The IUGR rate was analyzed using the Chi-square test. Data from 6 duplicate placental samples were analyzed as a 2 × 2 factorial treatment arrangement using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC, USA) unless otherwise specified (also analyzed using unpaired Student's *t*-test). The model utilized included the main factors of piglet birth weight (factor 1 = IUGR), adenosine supplementation (factor 2 = Ado) and their interaction (IUGR × Ado). Differences were considered significant at $P < 0.05$, and a tendency was considered at $0.05 \leq P < 0.1$.

## 3. Results

### 3.1. Characteristics of piglets

As shown in Fig. 1, the Ado group was higher than the Con group for piglet mean birth weight ($P < 0.001$) and placental efficiency ($P < 0.01$) (Fig. 1A and B), but had a lower IUGR rate ($P < 0.01$) (Fig. 1C).

### 3.2. Placental vessel density

Fig. 2 shows the immunostaining results of placental vascular density and VCAM1 expression in Normal and IUGR fetuses in the Con and Ado groups. As shown in the figure, the placenta from normal piglets had higher placental vascular density ($P < 0.01$) and VCAM1 expression ($P < 0.01$) compared to the placenta from IUGR piglets. Adenosine treatment also increased the placental vascular density ($P < 0.01$) and the expression level of VCAM1 ($P < 0.01$). Moreover, an interaction effect was observed between IUGR and Ado in their effect on VCAM1 expression ($P < 0.01$).

### 3.3. mRNA abundance of placental angiogenesis

The effects of adenosine and birth weight of newborn piglets on placental angiogenesis were further explored by analyzing the mRNA expression of angiogenesis-related genes. A total of 7 angiogenesis-related genes were evaluated by qRT-PCR (Fig. 3). The IUGR placenta had a marked decrease in the mRNA expression of *VEGF-A* ($P < 0.01$) and *TGF-1β* ($P < 0.05$). The adenosine treatment had an up-regulated trend in the mRNA expression of *Ang* ($P = 0.06$). Moreover, an interaction effect was observed in *VEGF-A* and *TGF-1β* mRNA expression between IUGR and Ado ($P < 0.05$). Interestingly, in *t*-test analysis, the mRNA levels of *VEGF-A* and *Ang* in the IUGR placenta were decreased compared to the Normal placenta in the Con group ($P < 0.05$), and had a rescued trend in adenosine treatment ($P = 0.07$ and $P = 0.08$). However, in normal placenta, there was no difference in the mRNA levels of *VEGF-A* and *Ang* in adenosine treatment ($P > 0.05$).

### 3.4. Expression of adenosine receptors in placenta

Next, the expression level of adenosine receptors in the placenta for IUGR and normal piglets were evaluated. As shown in Fig. 4A–D, the IUGR factor had no significant effect on mRNA levels of placental adenosine receptors, but the *ADORA2A* expression level was decreased in the IUGR placenta in immunostaining analysis ($P < 0.01$). In addition, the mRNA expression of both *ADORA2A* ($P < 0.01$) and *ADORA2B* ($P < 0.05$) revealed an increase with adenosine treatment, and similar results were also found in immunostaining analysis in that the expression level of *ADORA2A* was increased with adenosine treatment ($P < 0.01$) (Fig. 4E and F). The results also showed an interaction effect between IUGR and Ado in the relative fluorescence density of *ADORA2A* ($P < 0.01$).

### 3.5. Protein levels of placental angiogenesis and adenosine receptors

Fig. 5 displays the protein levels of angiogenesis-related markers and adenosine receptors estimated by Western blotting. The results showed that the expression of the angiogenesis-related signaling pathway proteins p-Stat3 and p-Akt were decreased in the IUGR placenta ($P < 0.05$). Only ADORA2A had a lower protein level in the IUGR placenta ($P < 0.05$). Moreover, adenosine treatment notably increased the protein expression levels of VCAM-1 ($P < 0.01$), VEGF-A ($P < 0.05$) and Ang ($P < 0.01$). Similar results were also observed

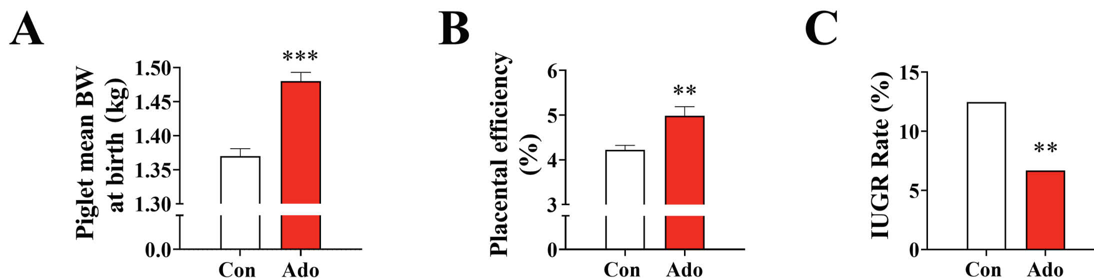


**Fig. 1.** Effects of maternal adenosine supplementation on the characteristics of piglets. (A) Piglet mean body weight (BW) at birth. (B) Placental efficiency = piglet weight (g)/placental weight (g). Data were analyzed by unpaired Student's *t*-test. (C) Intrauterine growth restriction (IUGR) rate was analyzed using the Chi-square test. The number of sows was 44 in each group. All data are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

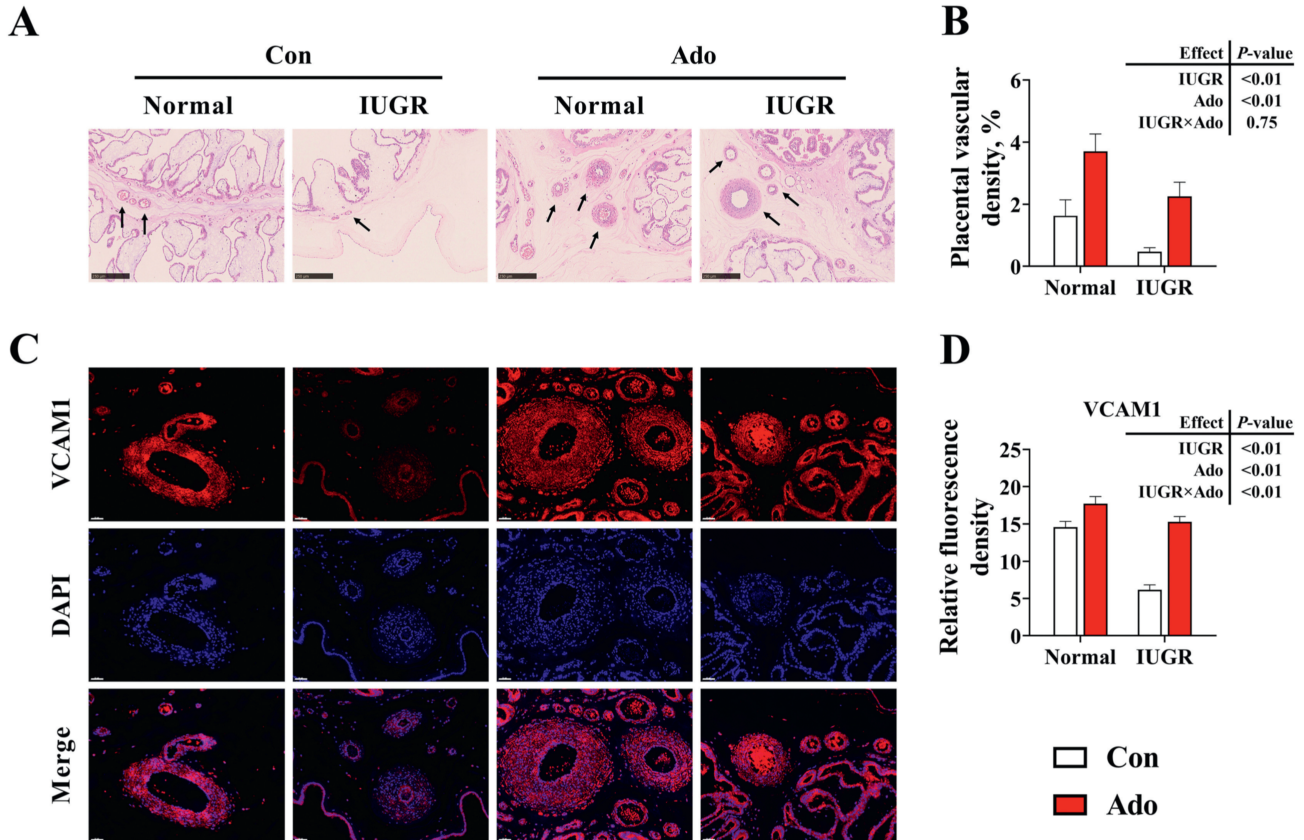


**Fig. 2.** Effects of maternal adenosine supplementation on the vascular density of placenta. (A, B) The H&E method was used to detect blood vessel density in the placenta, and the black arrows point to the placental blood vessels (bar = 250 µm, $n = 6$). (C, D) VCAM1 immunofluorescence staining in the placenta. (bar = 50 µm, $n = 6$). Statistical significance was determined by 2 × 2 factorial treatment arrangement, factor 1 = IUGR, factor 2 = Ado, interaction between factor 1 and 2 = IUGR × Ado. All data are presented as mean ± SEM. IUGR = intrauterine growth restriction; Ado = adenosine group; VCAM1 = vascular cell adhesion molecule-1.

in the protein expression of p-Stat3 and p-Akt ($P < 0.01$). Adenosine treatment also increased the protein expression of ADORA2A ($P < 0.01$) and ADORA3 ($P < 0.05$). An interaction effect was observed in p-Stat3 and p-Akt protein expression between IUGR and Ado ($P < 0.01$), as well as a trend in interaction effect on the protein level of VCAM1 ($P = 0.09$) and ADORA2A ($P = 0.06$).

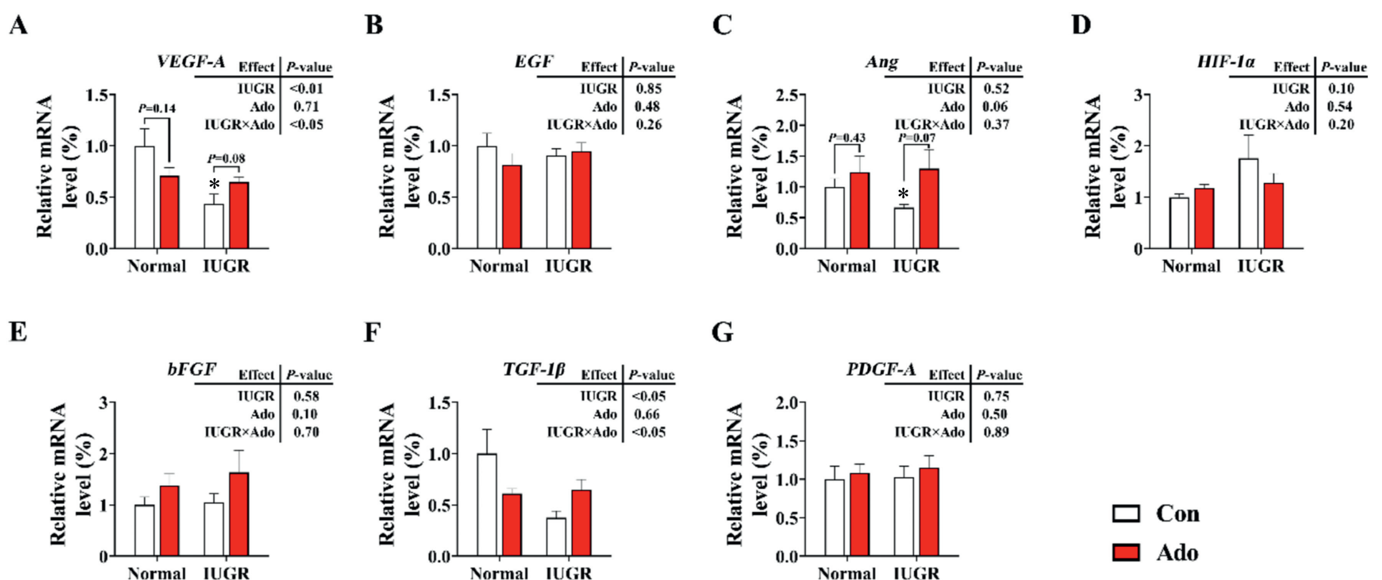


**Fig. 3.** Real-Time PCR mRNA expression analysis of angiogenesis-related factors in placenta. (A) *VEGF-A*, (B) *EGF*, (C) *Ang*, (D) *HIF-1α*, (E) *bFGF*, (F) *TGF-1β*, (G) *PDGF-A*. $n = 6$. Statistical significance was determined by 2 × 2 factorial treatment arrangement; factor 1 = IUGR; factor 2 = Ado; interaction between factor 1 and 2 = IUGR × Ado. All data are presented as mean ± SEM. The statistical significance in (A) and (C) were also determined by unpaired Student's *t*-test. $^{*}P < 0.05$, IUGR + Con vs. Normal + Con. IUGR = intrauterine growth restriction; Ado = adenosine group; *VEGF-A* = vascular endothelial growth factor A; *Ang* = angiogenin.

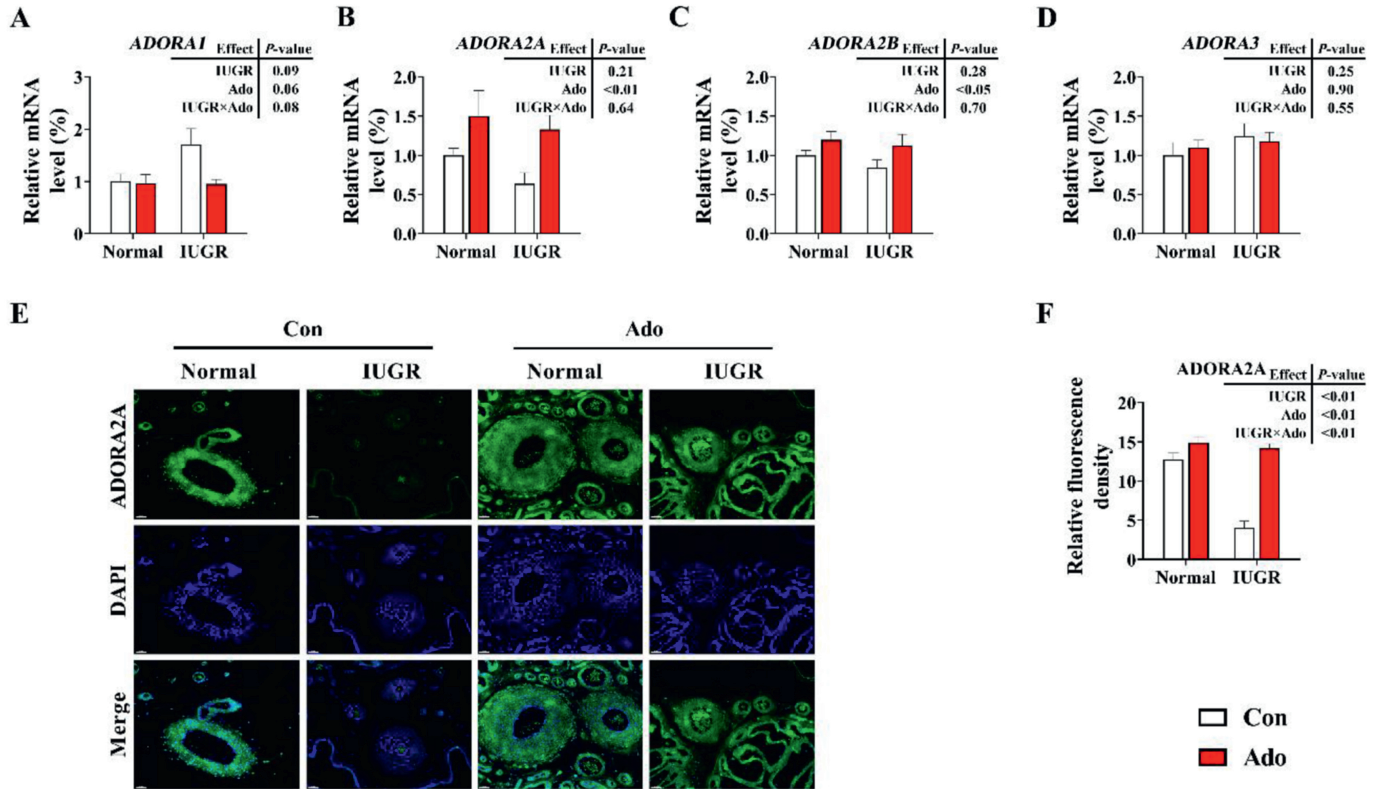


**Fig. 4.** The expression of adenosine receptors in the placenta. (A–D) Real-Time PCR mRNA expression analysis of the adenosine receptors in the placenta ($n = 6$). (E–F) ADORA2A immunofluorescence staining in the placenta (bar = 50 μm, $n = 6$). Statistical significance was determined by 2 × 2 factorial treatment arrangement; factor 1 = IUGR; factor 2 = Ado; interaction between factor 1 and 2 = IUGR × Ado. All data are presented as mean ± SEM. IUGR = intrauterine growth restriction; Ado = adenosine group.

## 4. Discussion

Previous studies have widely reported that abnormal placental angiogenesis is associated with IUGR occurrence (Hu et al., 2020; Tan et al., 2022), suggesting that improving the placental angiogenesis might alleviate adverse pregnancy outcomes (Huang et al., 2021a, 2021b). Despite previous reports about the potential of adenosine in promoting angiogenesis, the role of adenosine in placental angiogenesis remains unclear. Pigs, as animals commonly used in biomedical research on human pregnancy, have been favored due to the physiological similarities they have to humans (Bazer et al., 2012; Cai et al., 2018). In this study, we investigated whether maternal adenosine supplementation during pregnancy could improve pregnancy outcomes by detecting changes in placental angiogenesis. Our results demonstrated that adenosine supplementation could significantly increase the mean birth weight and placental efficiency in piglets, and the Ado group was shown to have a significantly lower IUGR rate than the Con group (6.84% vs 12.47%), suggesting the great potential of adenosine supplementation to ameliorate pregnancy outcomes in sows.

The placenta plays an important role in fetal growth (Reynolds et al., 2006; Zhao et al., 2020), and adequate placental angiogenesis is essential for successful pregnancy and optimal growth of the fetus (Gualdoni et al., 2021). Thus, to further explore the different effects of adenosine on Normal and IUGR fetuses, the placentae from IUGR and normal-weight (Normal) fetuses were separately collected as 2 groups for further analysis. The results showed that the IUGR placenta had significantly poorer angiogenesis, and adenosine treatment could significantly promote angiogenesis in the placenta (especially in the IUGR placenta), as well as restore the low expression of *VEGF-A* and *Ang* in the IUGR placenta. Notably, there was an interaction effect between IUGR and Ado in the pro-angiogenesis effect of adenosine, and adenosine had no significant effect on the mRNA expressions of angiogenesis-related genes in the Normal placenta, suggesting that the beneficial effects of adenosine treatment on IUGR occurrence may be attributed to the presence of poor angiogenesis in the IUGR placenta. These results were consistent with several previous studies reporting that (1) IUGR piglets had lower vessel density in the placenta compared to normal piglets, coupled with lower CD31 and VEGF-A level (Campos et al., 2012; Hu et al., 2020; Wang et al., 2017); (2) there was a close relationship between adenosine and embryonic development (Rivkees and Wendler, 2017); (3) adenosine signaling can be activated to exert biological effects, including but not limited to angiogenesis, by up-regulating the expression of angiogenesis factors such as VEGF, TGF-1β and Ang (Bahreyni et al., 2018; Gorain et al., 2019; Zhang et al., 2019).

Differential binding to adenosine receptors may be critical for adenosine activity and its biological effects (Salsoso et al., 2017). In this study, the mRNA level of 4 adenosine receptors in the placenta was further evaluated. Interestingly, only the expression of ADORA2A showed a significant increase under adenosine treatment both in the Normal and IUGR placenta. Although the mRNA level of *ADORA2B* trended toward a similar expression with *ADORA2A*, the changes in *ADORA2B* (up-regulated only 20% after adenosine treatment) were much less than *ADORA2A* (up-regulated nearly 50% after adenosine treatment). Further, immunostaining analysis also showed that the expression of ADORA2A was simultaneously affected by IUGR and Ado, as well as their interaction effects. These results seemed to imply that adenosine was more

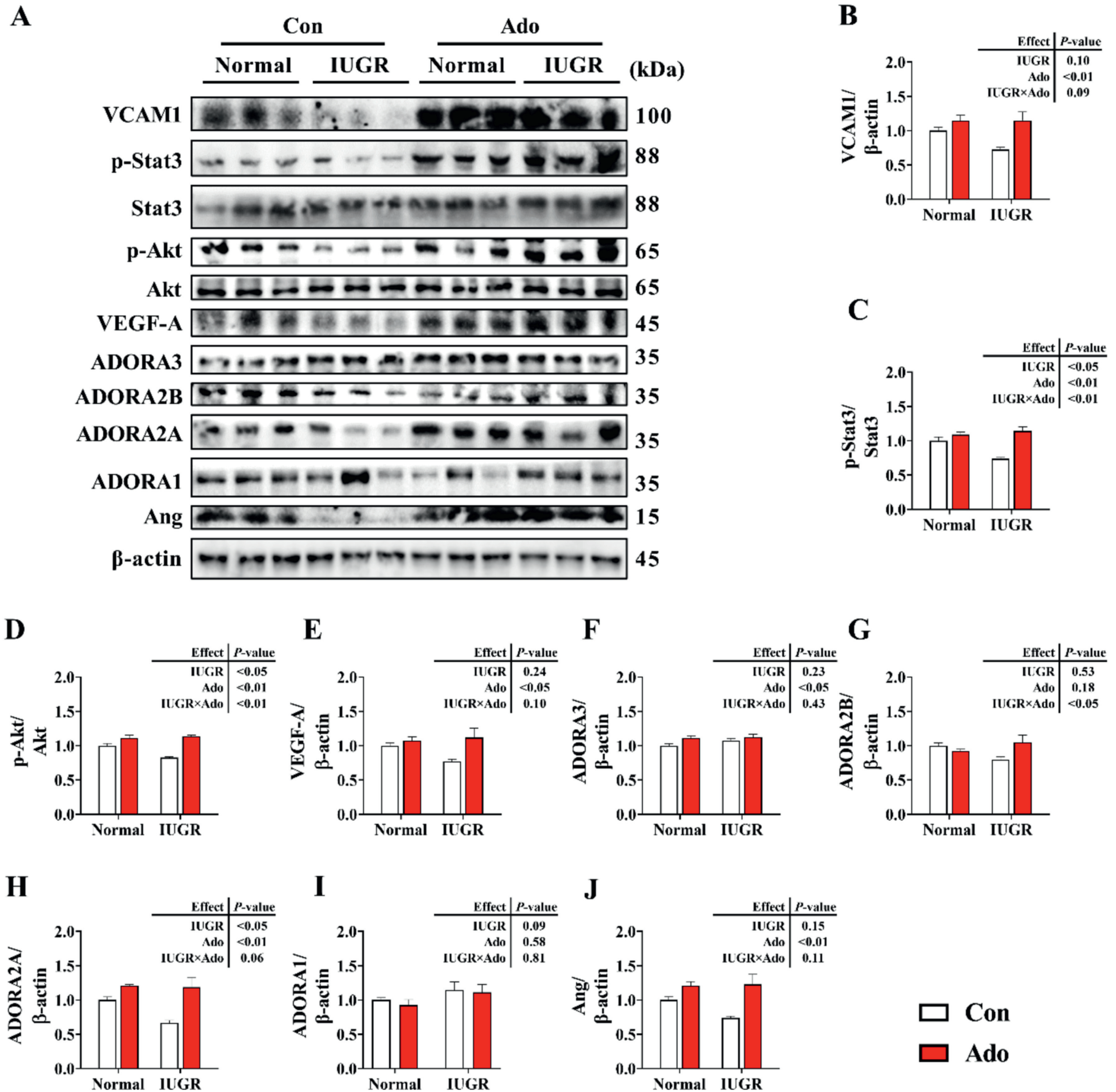


**Fig. 5.** Western blot protein expression analysis of angiogenesis-related factors (B, VCAM1; C, p-Stat3; D, p-Akt; E, VEGF-A; J, Ang) and adenosine receptors (F, ADORA3; G, ADORA2B; H, ADORA2A; I, ADORA1) in the placenta. $n = 6$. Statistical significance was determined by $2 \times 2$ factorial treatment arrangement; factor 1 = IUGR; factor 2 = Ado; interaction between factor 1 and 2 = IUGR × Ado. All data are presented as mean ± SEM. IUGR = intrauterine growth restriction; Ado = adenosine group.

likely to increase ADORA2A level to promote angiogenesis in the IUGR placenta. The promoting effect of ADORA2A on angiogenesis has been demonstrated in previous studies (Acurio et al., 2017; Liu et al., 2017). Collectively, adenosine supplementation can up-regulate ADORA2A level and improve angiogenesis in the IUGR placenta.

Furthermore, the protein expression of angiogenesis-related markers and adenosine receptors, as well as the possible underlying mechanisms were explored. Only ADORA2A was found to have the same expression trend as its mRNA expression (which ADORA2B did not have), which further suggested the important role ADORA2A plays in placental angiogenesis promoted by adenosine. Similar results were also observed in angiogenesis-related protein expression. Interestingly, the angiogenesis-related signaling pathway Stat3 and Akt showed a similar expression trend with angiogenesis markers. Previous studies also reported that Stat3 is an important regulator in angiogenesis (Pereira et al., 2015), and our previous study also demonstrated the ability of Stat3 in improving placental angiogenesis (Hu et al., 2021; Huang et al., 2021a). As the downstream signaling target of adenosine, Akt also plays an important role in angiogenesis as previously reported (Azambuja et al., 2019; Liu et al., 2017).

## 5. Conclusions

Impaired angiogenesis in the placenta during pregnancy was shown to be associated with the occurrence of IUGR piglets, while maternal adenosine supply of 1 g/kg during the gestation of sows could promote angiogenesis in placentae (especially in IUGR placentae), finally increase the piglet birth weight and reduce the IUGR rate. The underlying mechanism for the positive effects of adenosine might be linked to the activation of ADORA2A and Stat3/Akt signaling.

## Author contributions

**Chengquan Tan:** Conceptualization, Methodology, Writing - Review & Editing, Project administration. **Hefeng Luo:** Methodology, Investigation, Writing - Original Draft. **Zifang Wu:** Investigation, Data Curation, Writing - Original Draft. **Jiawei Nie:** Investigation, Data Curation. **Deyuan Wu:** Investigation. **Shuangbo Huang:** Investigation. **Jianzhao Chen:** Investigation. **Huangjin Liang:** Resources. **Xiangyu Hao:** Resources. **Li Feng:** Resources.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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## Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.02.003.

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