Daidzein Supplementation Enhances Embryos Survival by Improving Hormones, Antioxidant Capacity, and Metabolic Profiles of Amniotic Fluid in Sows

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Abstract

Background: Daidzein (DAI) is a kind of natural isoflavonic phytoestrogen with estrogenic activity. However, little is known about its influence on early fetal growth in mammalian animals. In this study, we investigated the effects dietary DAI supplementation on early fetal development in sows. To explore the potential mechanisms, the metabolic profiles of amniotic fluid collected at 35 days of gestation (dg) was determined by using metabolomics.

Results: Results show that DAI supplementation at a dose of 200 mg/kg significantly enhanced the number of viable embryos at the early gestation stage ($P < 0.05$). DAI significantly elevated the concentrations of estrogen (E) and insulin-like growth factor-I (IGF-I) in the amniotic fluid ($P < 0.05$). Moreover, DAI tended to increase the concentration of progesterone, but decrease the concentration of tumor necrosis factor α (TNF-α) in the amniotic fluid ($0.05<P<0.10$). Interestingly, the activity of glutathione peroxidase (GSH-Px) was higher in the DAI group than in the CON group ($P < 0.05$). An ^{1}H NMR-based metabolomics analysis identified a number of metabolites in the amniotic fluid, and some critical metabolites such as arginine, creatine, and citrate were found to be significantly elevated upon DAI supplementation ($P < 0.05$). Importantly, the metabolic pathways involved in arginine and proline metabolisms were found to be significantly affected by DAI.

Conclusions: These findings suggested that dietary DAI supplementation may improve embryos survival by improving hormones, antioxidant capacity, and metabolic profiles in the maternal amniotic fluid.

Background

Embryo loss or fetal death during pregnancy is one of the major factors that decrease the reproductive performance of mammalian animals. Early pregnancy loss (EPL) is one of the most common complications of pregnancy, which occurs at the early gestation period [1, 2]. Currently, EPL-induced pregnancy ending accounts for nearly 15–20% of all pregnancies [3]. In addition to congenital defects (e.g. chromosomal abnormalities), a wide variety of factors such as endocrine disorders, infections, autoimmune diseases, and hypotrophy can cause the EPL [4–6].

The reproductive activities and fetal development for mammalian animals are regulated by a number of hormones and growth factors. Accumulated evidences showed that estrogen is the main regulator of placental trophoblast cell function during mammalian pregnancy, and decrease in serum estrogen concentration may lead to developing of the EPL [7–9]. For instance, estrogens can serve as potent vasodilators that can increase the blood flow in the uterine-placental region [10]. Moreover, estrogens were found to stimulate early placental angiogenesis through upregulating of the vascular endothelial growth factor (VEGF) [11]. Previous study indicated that conversion of androstenedione (A4) to estrone (E1) and testosterone (T0) to 17β-estradiol (E2) are catalyzed by placenta aromatase [12]. However, a transient expression of cytochrome P450 aromatase in porcine placenta during early gestation period may result in decreased production of estrogens in pigs [13].

Daidzein (7,4'-dihydroxyisoflavone, DAI) is a class of non-steroidal polyphenolic secondary metabolites, which has attracted considerable research interests because of their antioxidant, anti-inflammatory, and differentiation-inducing abilities [14–16]. Importantly, it can directly bind to estrogen receptor subtypes (i.e., ERα and ERβ) to exert estrogenic and/or antiestrogenic effects [17]. Extensive studies have focused on the role of daidzein on animal growth and health [18]. However, accumulating evidences showed that daidzein may have a beneficial effect on animal fertility during pregnancy. For instance, previous study demonstrated that rats supplemented with 50 mg/kg daidzein from gestation until delivery stage resulted in increased the total litter weight and the total viable newborn weight [19]. Interestingly, daidzein has been identified in mammalian amniotic fluids which not only offers evidence of transfer from the mother to the fetus [20, 21], but also suggests that daidzein may play a critical role in regulating embryos development. Although the role of daidzein supplementation in reproductive performance has been partly investigated [22, 23], the protective effects and mechanisms of daidzein on the early embryo development are still unclear.

In the present study, we explored the effects of daidzein supplementation on early embryos development in sows. To gains insights into the mechanisms of DAI-regulated embryo development, the immunoglobulins, antioxidant parameters, and hormones of in the amniotic fluid have also been investigated. Moreover, an ^{1}H-NMR-based metabolomics analysis was utilized to explore the profiles of metabolites in the amniotic fluid, which may also provide novel insights into the role of daidzein supplementation in the regulation of early embryo development.

Methods

Animals and housing

The experimental protocol was performed in accordance with guidelines established by the Animal Management Rules of the Ministry of Health of the People's Republic of China. In addition, our study protocol was approved by the Animal Care and Use Committee of Sichuan Agricultural University (No. 20190318). The experiment was conducted at a commercial pig farm (Guangyuan, Sichuan province, China).

A total of 120 multiparous Yorkshire × Landrace sows (3–5 of parity) were randomly and equally divided into control (CON) and daidzein (DAI) groups. After confirming that all sows were in the oestrous stage, the sows were artificially inseminated twice with unfrozen semen within two days. The sows were housed in individual gestation stalls (2.20 × 0.65 m) from day 1 of mating to the day 34 of gestation with free access to drinking water. The ambient temperature was maintained at between 20 and 25 °C. During the feeding period, special attention was paid to the ventilation and tidiness of the accommodations.

Animal Treatment and Feeding

The experiment began on day 1 of gestation and ended on day 34 of gestation. During this period, the sows of control group were fed with a basal diet, and the DAI group was fed with a basal diet plus an

extra 0.02% daidzein (Sichuan Jun Zheng Bio-Feed Co., Ltd., Chengdu, China). The basal diet was formulated on the basis of the nutrition needs of pregnant sows according to the National Research Council (NRC, 2012), and their compositions are shown in Supplementary Table 1. Sows were given 2.3 kg diets per day and fed twice per day (08:00 and 15:00 hours) throughout the experiment.

Sample and data collection

A random subset of sows (n = 6 per treatment) with close average body weight were selected at day 35 of gestation. The sows were then killed to obtain uteri, and the uteri were opened longitudinally along the anti-mesometrial side to obtain embryos from the attachment sites. A volume of 5 mL amniotic fluid from each embryo was harvested with sterile syringes. After all amniotic fluid samples were centrifuged at 2000 × g for 10 min at 4 °C to remove meconium, the samples were stored at − 80 °C until metabolomics and biochemical assays. Meanwhile, number of viable or mummy fetuses, average weight of viable fetuses, and size (crown-to-rump length) of viable fetuses were recorded as previously described [24].

Amniotic fluid reproductive hormones

Hormones including estrogen (E), progesterone (P), leptin (LEP) and insulin-like growth factor-1 (IGF-1) in the amniotic fluid were analyzed using commercially available porcine ELISA kits (Beijing Donggeboye Biological Technology Co., Ltd., Beijing, China), and the detailed operations were as per the kits' instructions. The sensitivity and intra- and inter-assay coefficients of variation for E, P, LEP, and IGF-1 were listed in Supplementary Table 2.

Amniotic fluid immunoglobulin levels

The amniotic fluid samples from sows were assessed for immunoglobulin levels of immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) with commercial ELISA kits (Minneapolis, MN, USA) according to the manufacturer's instructions. A SpectraMax M2 spectrophotometer (Molecular Devices, CA, USA) was used to measure standards and samples at optical density values of 700 nm (IgG) or 340 nm (IgA, IgM). The concentration of immunoglobulin was calculated using standard curve and was expressed as μg per millilitre of amniotic fluid.

Quantification of cytokines in amniotic fluid

Concentrations of interferon γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-10 (IL-10) were analyzed using porcine commercial ELISA kits purchased from R&D system. Assays were performed on the Immulon 2 HB 96-well plates which were coated with corresponding anti-porcine cytokines. Samples were added to the wells in a volume of 50 μl plus 50 μl of PBS–1% BSA and incubated for 2 h at room temperature (RT). The reaction was amplified with biotinylated monoclonal antibodies to porcine IFN-γ (1 μg/ml), TNF-α (250 μg/ml), IL-1 (0.1 μg/ml), IL-6 (0.2 μg/ml), and IL-10 (100 μg/ml). Plates were incubated for 1 h at RT. Detection was carried out with peroxidase-conjugated streptavidin (1:5000; Jackson Laboratories) following 60 min of incubation at RT, and the reaction was visualized with PNPP (Sigma-Aldrich). The assays were analyzed colorimetrically using a SpectraMax M2 spectrophotometer (Molecular Devices, CA, USA).

Antioxidant parameters of sows' amniotic fluid

All antioxidant-related kits from Nanjing Jiancheng Bioengineering Institute were used to determine the antioxiant parameters according to the manufacturers' instructions. Amniotic fluid antioxidant status was measured using a SpectraMax M2 spectrophotometer (Molecular Devices, CA, USA). Malondialdehyde (MDA) was measured using an established thiobarbituric acid (TBARS) method [25]. MDA, a thiobarbituric acid reactive substance (TBARS), reacts with thiobarbituric acid (TBA) to form a 1: 2 MDA-TBA adduct that is absorbed at 548 nm. The concentration of MDA was calculated using standard curve and was expressed as nmol per millilitre of amniotic fluid.

The SOD activity was measured by using the procedure reported by Thomas [26]. The SOD activity was assayed by reacting with 2-(4-iodophenyl)3-(4-nitrophenol)-5-phenyltetrazolium chloride to generate red formazan which could be spectrophotometrically determined at 550 nm. The content of SOD activity was determined as described by the manufacture' instructions, which was expressed as U per millilitre of amniotic fluid.

Catalase (CAT) activity was measured spectrophotometrically at 620 nm using a previous described method [27]. The method is based on the fact that the dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide with the formation of perchloric acid as an unstable intermediate. CAT activity was expressed as U per millilitre of amniotic fluid, where one unit is defined as the amount that decreases 1 mmol/L H_2O_2 within 1 min per milliliter of amniotic fluid.

Glutathione peroxidase (GSH-Px) activity was measured using a colorimetric method described by Rotruck et al. [28]. The enzymatic reaction was terminated by the addition of 5-50-dithiobis-(2-nitrobenzoic acid) (DTNB) (80 mg in 1% sodium citrate), which generated a light-yellow composite that could be measured at 412 nm. The GSH concentration in the experimental samples was extrapolated from the standard curve. In addition, GSH concentration was expressed as mg per millilitre.

The Total antioxidant capacity (T-AOC) activity was measured in accordance with the method of Prieto et al. [29]. The reaction was assayed by the reduction of Fe^{3+}- tripyridyltriazine to Fe^{2+}- tripyridyltriazine and could be measured at 405 nm. T-AOC was expressed as U per milliliter, where one U represents the 0.01 increase in the absorbance value in 1 minute per millilitre.

Sample preparation and ^{1}H-NMR measurement

For NMR analysis, amniotic fluid samples were left to thaw, and aliquots of 200 μL were mixed with 400 μL phosphate buffer (0.045 M NaH_2PO_4/K_2HPO_4, pH 7.4, 100% D_2O). After centrifugation (12 000 × g, 10 min), an aliquot of 550 μL of each sample supernatant was subsequently transferred to 5-mm ^{1}H-NMR tubes (Norell, Landisville, NJ, USA). The pure metabolite molecules used for referencing were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

The NMR spectra of amniotic fluid samples were recorded at 298K using an Agilent DD2 600 MHz NMR spectrometer (Agilent Technologies, Inc., CA, USA). A standard ^{1}H-NMR spectrum with water suppression using a standard NOESY pulse sequence (recycle delay – G1 – 90°–t_1 – 90°–t_m–G2 – 90°–acquisition). For each sample, parameters were set as follows: 128 scans were collected with a relaxation delay of 3 s; acquisition time of 1.71 s; TD of 32 k; and SW of 16 ppm. All NMR spectra were processed with a line broadening of 1 Hz and corrected for phased and baseline distortions using Topspin 3.0 (Bruker Biospin). The chemical shifts in amniotic fluid spectra were referenced to the anomeric proton signal of α-glucose at δ 5.23. Finally, the spectra-consistent 32,000 data points were normalized using the Probabilistic Quotient Normalization (PQN) method. The metabolite assignments were usually obtained by considering chemical shifts, coupling constants and relative intensities.

^{1} H-NMR spectroscopic analysis

For statistical analysis, reduction of ^{1}H-NMR spectrum (9.0–0.5 ppm) was performed using MestReNova (Mestrelab Research). All NMR spectra were reduced to 1700 integral segments with an equal width of 0.004 ppm. Before data analysis, each amniotic fluid sample ^{1}H-NMR spectrum was further removed the water (5.5–4.4 ppm) and urea (6.1–5.5 ppm) regions to eliminate the effects of variation in the suppression of the water and urea signals.

The SIMCA-P^{+} software (V14.0, Umetrics AB, Umea, Sweden) was facilitated for multivariate statistical analysis. For primary visualization, distribution and clustering, the principal component analysis (PCA) model was applied to reveal differences with biological significance. Following this, the partial least squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed using a unit variance-scaled approach at a confidence level of 95%. To minimize the impact of the high variability in the metabolite measurements, unit variance scaling was utilized. R^2X and R^2Y, the fraction of variation that the model explains in the independent variables (X) and dependent variables (Y) and the predictive accuracy of the model (Q^2Y), were estimated by the PLS-DA cross validation. The quality of the calculated model was accessed using a 200-iteration permutation test.

Potential differential metabolite selection was based on loading plot and variable importance in the projection (VIP), where only values with VIP > 1 and Bonferroni-corrected (Correlation Coefficient) $P < 0.05$ were considered statistically significant. The heat map and hierarchical cluster analysis (HCA) of differential metabolites were performed using the MeV software package (version 4.9.0). Finally, for identification of the most altered metabolic pathways, a set of significantly altered metabolites was used as the input for KEGG Pathway Analysis (http://www.kegg.com/).

Statistical analysis

Statistical analyses including all amniotic fluid antioxidant and immune parameters, as well as foetal data were performed using the Student's t-test of SAS 9.0 (SAS Institute, Cary, NC, USA). Each sow was

considered as a statistical unit. Data was presented as means ± standard deviations (SD). *P*-values less than 0.05 were considered statistically significant.

Results

Influence of daidzein on early embryo development in sows

As compared to the CON group, daidzein supplementation significantly increased the number of viable embryos ($P < 0.01$) at 35 dg (Fig. 1). Moreover, the number of mummy embryos was significant lower in the DAI group than in the control group ($P < 0.05$). There were no significant differences in the size and average weight of viable embryos between the two groups ($P > 0.05$).

Influence of daidzein on reproductive hormones and growth-related factors in amniotic fluid

The concentrations of critical hormones and growth factors in the amniotic fluid are shown in Fig. 2. As compared to the CON group, daidzein supplementation significantly increased the concentrations of estrogen and IGF-I in the amniotic fluid ($P < 0.05$). Moreover, daidzein supplementation tended to increase the concentration of progesterone in the amniotic fluid ($P = 0.09$). However, there was no obvious differences in the activity of leptin between two groups (Fig. 2).

Influence of daidzein on immunoglobulins and proinflammatory cytokines in amniotic fluid

As show in Table 1, dietary supplementation of 200 mg/kg daidzein had no significant influence on the concentrations of immunoglobulins such as the IgA, IgG, and IgM between the two groups ($P > 0.05$). Moreover, there were no significant differences in the concentrations of proinflammatory cytokines such as the IFN-γ, IL-1, IL-6, and IL-10. However, daidzein supplementation tended to decrease IgM concentration in the amniotic fluid ($P = 0.08$).

Table 1

Effects of daidzein supplementation on the amniotic fluid immune responses of sows[a]

	Treatment[b]		
Items[c]	CON	DAI	P-value*
IgA (μg/mL)	44.63 ± 6.79	44.88 ± 3.50	0.936
IgG (μg/mL)	1069.07 ± 17.72	1030.27 ± 166.18	0.582
IgM (μg/mL)	111.33 ± 10.58	96.47 ± 15.41	0.080
IFN-γ (pg/ml)	2165.07 ± 210.20	2135.03 ± 444.65	0.884
IL-1 (ng/L)	98.66 ± 18.25	99.97 ± 19.22	0.906
IL-6 (ng/L)	650.32 ± 120.52	585.29 ± 174.44	0.470
IL-10 (ng/L)	92.94 ± 27.22	97.54 ± 31.54	0.793
TNF-α (pg/ml)	259.95 ± 74.35	180.42 ± 74.81	0.095

[a] Values are means with standard deviations, n = 6.

[b] CON: A corn-soybean basal diet; DAI: A basal diet supplemented with 200 mg/kg daidzein.

[c] IgA: Immunoglobulin A; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IFN-γ: Interferon-γ; IL-1: Interleukin 1. IL-6: Interleukin 6; IL-10: Interleukin 10; TNF-α: Tumour necrosis factor α.

* P-value < 0.05 represents significant differences in mean values according to the results of t-test.

Influence of daidzein on antioxidant indicators of amniotic fluid

As shown in Table 2, dietary supplementation of 200 mg/kg daidzein had no significant influence on activities of CAT, MDA, SOD and T-AOC in the amniotic fluid ($P > 0.05$). However, daidzein supplementation significantly increased the activity of GSH-Px in the amniotic fluid ($P < 0.05$).

Table 2

Effects of daidzein supplementation on the amniotic fluid antioxidant status of sows[a]

	Treatment[b]		
Items[c]	CON	DAI	P-value*
T-AOC (U/mL)	5.24 ± 0.55	5.73 ± 0.50	0.136
SOD (U/mL)	80.55 ± 5.25	86.61 ± 7.36	0.131
CAT (U/mL)	19.91 ± 2.78	19.51 ± 3.00	0.818
GSH-Px (U/mL)	179.63 ± 9.17	269.89 ± 17.70	< 0.01
MDA (nmol/mL)	2.97 ± 0.57	2.60 ± 0.53	0.278

[a] Values are means with standard deviations, n = 6.

[b] CON: A corn-soybean basal diet; DAI: A basal diet supplemented with 200 mg/kg daidzein.

[c] T-AOC: Total antioxidant capacity; SOD: Superoxyde dismutase; CAT: Catalase; GSH-Px: glutathione peroxidase; MDA: Malondialdehyde.

* *P*-value < 0.05 represents significant differences in mean values according to the results of t-test.

^{1}H-NMR spectra and identification of metabolites in amniotic fluid

Typical standard ^{1}H NMR spectra of amniotic fluid with annotations on the identified metabolites are depicted in Fig. 3. For the amniotic fluid samples, a total of 38 metabolites were quantified. The average concentration of the metabolites, as determined using Student's t-test, is listed in Table 3. The levels of most metabolites, including those of amino acids and their derivatives (Alanine, Glutamine, Phenylalanine, Glutathione, Leucine, Methionine, Asparagine, Isoleucine, Valine, Lysine, Taurine, Ornithine, Arginine), carbohydrates and their derivatives (Glucose, Ethanol, Citrate, Methanol, Ethanolamine), sn-Glycero-3-phosphocholine, myo-Inositol, Urea, Formate, 2-hydroxyisocaproate, Trimethylamine N-oxide, Creatine, increased, While the levels of Tyrosine, Hypoxanthine, Fucose, Fructose, Lactate, Fumarate, Choline, Glycine, Malate, and O-Phosphocholine decreased in the DAI-treated group compared to the control group. Therefore, the levels of 25 metabolites increased, while the levels of 11 metabolites decreased in the DAI group compared to the CON group.

Table 3
Quantitative comparison of amniotic fluid metabolites from DAI and CON sows.

Metabolite	Chemical Shift (ppm)	DAI Integrals (Mean ± SD)	CON Integrals (Mean ± SD)	Ratio DAI/CON	*p*-Value
Hypoxanthine	8.21	$3.65 \times 10^{-5} \pm 1.38 \times 10^{-5}$	$5.84 \times 10^{-5} \pm 1.17 \times 10^{-5}$	0.63	0.01
Tyrosine	6.93	$1.47 \times 10^{-5} \pm 4.92 \times 10^{-6}$	$2.47 \times 10^{-5} \pm 8.06 \times 10^{-6}$	0.60	0.03
Lactate	4.11	$7.53 \times 10^{-3} \pm 4.74 \times 10^{-4}$	$8.49 \times 10^{-3} \pm 5.29 \times 10^{-4}$	0.89	0.008
Fructose	4.10	$8.67 \times 10^{-4} \pm 6.85 \times 10^{-5}$	$9.87 \times 10^{-4} \pm 9.07 \times 10^{-5}$	0.88	0.03
Fucose	4.09	$2.28 \times 10^{-3} \pm 1.86 \times 10^{-4}$	$2.63 \times 10^{-3} \pm 1.84 \times 10^{-4}$	0.87	0.008
Arginine	3.23	$1.56 \times 10^{-3} \pm 2.62 \times 10^{-4}$	$1.23 \times 10^{-3} \pm 2.51 \times 10^{-4}$	1.27	0.049
Creatine	3.04	$3.88 \times 10^{-3} \pm 5.47 \times 10^{-4}$	$3.20 \times 10^{-3} \pm 2.76 \times 10^{-4}$	1.22	0.02
Citrate	2.69	$7.53 \times 10^{-4} \pm 1.31 \times 10^{-4}$	$5.89 \times 10^{-4} \pm 1.19 \times 10^{-4}$	1.28	0.046
Isoleucine	1.95	$4.49 \times 10^{-4} \pm 4.80 \times 10^{-5}$	$4.12 \times 10^{-4} \pm 6.51 \times 10^{-5}$	1.09	0.28
2-hydroxyisocaproate	1.73	$1.39 \times 10^{-3} \pm 1.68 \times 10^{-4}$	$1.26 \times 10^{-3} \pm 2.00 \times 10^{-4}$	1.10	0.25
Leucine	1.71	$4.86 \times 10^{-4} \pm 6.81 \times 10^{-5}$	$4.64 \times 10^{-4} \pm 5.54 \times 10^{-5}$	1.05	0.57
Valine	2.27	$2.88 \times 10^{-4} \pm 4.05 \times 10^{-5}$	$2.64 \times 10^{-4} \pm 4.22 \times 10^{-5}$	1.09	0.35

The selected Nuclear Magnetic Resonance (NMR) peaks (chemical shifts in the second column) determined in the amniotic fluid ^{1}H-NMR spectra for each group, were used for the quantification of metabolites, reported as mean and relative standard deviation. Results were validated by the univariate t-test.

Metabolite	Chemical Shift (ppm)	DAI Integrals (Mean ± SD)	CON Integrals (Mean ± SD)	Ratio DAI/CON	*p*-Value
Isobutyrate	2.38	$1.76 \times 10^{-4} \pm 2.92 \times 10^{-5}$	$1.75 \times 10^{-4} \pm 3.82 \times 10^{-5}$	1.00	0.99
Ethanol	3.66	$3.30 \times 10^{-3} \pm 2.02 \times 10^{-4}$	$3.18 \times 10^{-3} \pm 3.46 \times 10^{-4}$	1.04	0.5
Lysine	3.03	$1.18 \times 10^{-3} \pm 1.80 \times 10^{-4}$	$1.07 \times 10^{-3} \pm 1.87 \times 10^{-4}$	1.11	0.31
Alanine	3.78	$2.14 \times 10^{-3} \pm 1.23 \times 10^{-4}$	$2.11 \times 10^{-3} \pm 1.44 \times 10^{-4}$	1.01	0.72
Glutamine	3.77	$1.97 \times 10^{-3} \pm 1.04 \times 10^{-4}$	$1.96 \times 10^{-3} \pm 9.41 \times 10^{-5}$	1.01	0.84
Methionine	2.65	$2.52 \times 10^{-4} \pm 3.54 \times 10^{-5}$	$2.34 \times 10^{-4} \pm 2.32 \times 10^{-5}$	1.08	0.32
Glutathione	3.74	$1.18 \times 10^{-3} \pm 1.44 \times 10^{-4}$	$1.15 \times 10^{-3} \pm 1.28 \times 10^{-4}$	1.03	0.7
Pyruvate	3.28	$4.30 \times 10^{-3} \pm 4.06 \times 10^{-4}$	$4.38 \times 10^{-3} \pm 4.33 \times 10^{-4}$	0.98	0.74
Malate	2.68	$1.17 \times 10^{-4} \pm 2.18 \times 10^{-5}$	$1.21 \times 10^{-4} \pm 2.49 \times 10^{-5}$	0.97	0.77
Asparagine	4.00	$7.56 \times 10^{-3} \pm 5.40 \times 10^{-4}$	$7.01 \times 10^{-3} \pm 8.62 \times 10^{-4}$	1.08	0.21
Ornithine	3.06	$2.94 \times 10^{-4} \pm 5.17 \times 10^{-5}$	$2.60 \times 10^{-4} \pm 6.35 \times 10^{-5}$	1.13	0.34
Phenylalanine	3.27	$4.26 \times 10^{-3} \pm 5.20 \times 10^{-4}$	$4.24 \times 10^{-3} \pm 4.25 \times 10^{-4}$	1.01	0.93
Histidine	3.98	$9.35 \times 10^{-4} \pm 1.06 \times 10^{-4}$	$9.34 \times 10^{-4} \pm 5.65 \times 10^{-5}$	1.00	0.98

The selected Nuclear Magnetic Resonance (NMR) peaks (chemical shifts in the second column) determined in the amniotic fluid ^{1}H-NMR spectra for each group, were used for the quantification of metabolites, reported as mean and relative standard deviation. Results were validated by the univariate t-test.

Metabolite	Chemical Shift (ppm)	DAI Integrals (Mean ± SD)	CON Integrals (Mean ± SD)	Ratio DAI/CON	*p*-Value
Ethanolamine	3.82	$3.11 \times 10^{-3} \pm 3.43 \times 10^{-4}$	$2.97 \times 10^{-3} \pm 3.41 \times 10^{-4}$	1.05	0.49
Choline	4.07	$5.08 \times 10^{-3} \pm 7.16 \times 10^{-4}$	$5.26 \times 10^{-3} \pm 4.86 \times 10^{-4}$	0.96	0.61
O-Phosphocholine	4.17	$2.18 \times 10^{-4} \pm 5.45 \times 10^{-5}$	$2.25 \times 10^{-4} \pm 6.53 \times 10^{-5}$	0.97	0.86
sn-Glycero-3 phosphocholine	3.95	$6.35 \times 10^{-4} \pm 3.90 \times 10^{-5}$	$6.20 \times 10^{-4} \pm 1.28 \times 10^{-5}$	1.03	0.37
Trimethylamine N-oxide	3.24	$6.80 \times 10^{-4} \pm 1.00 \times 10^{-4}$	$6.11 \times 10^{-4} \pm 1.34 \times 10^{-4}$	1.11	0.33
Taurine	3.26	$5.46 \times 10^{-4} \pm 9.17 \times 10^{-5}$	$4.87 \times 10^{-4} \pm 3.79 \times 10^{-5}$	1.12	0.16
myo-Inositol	3.62	$1.75 \times 10^{-3} \pm 1.41 \times 10^{-4}$	$1.69 \times 10^{-3} \pm 1.39 \times 10^{-4}$	1.03	0.52
Methanol	3.36	$5.77 \times 10^{-3} \pm 1.14 \times 10^{-3}$	$5.65 \times 10^{-3} \pm 3.61 \times 10^{-4}$	1.02	0.81
Glucose	3.94	$7.91 \times 10^{-4} \pm 4.63 \times 10^{-5}$	$7.59 \times 10^{-4} \pm 2.85 \times 10^{-5}$	1.04	0.18
Glycine	3.56	$4.13 \times 10^{-3} \pm 2.03 \times 10^{-4}$	$4.30 \times 10^{-3} \pm 4.36 \times 10^{-4}$	0.96	0.43
Fumarate	6.92	$2.22 \times 10^{-5} \pm 2.74 \times 10^{-6}$	$2.49 \times 10^{-5} \pm 9.07 \times 10^{-6}$	0.89	0.5
Formate	8.46	$3.70 \times 10^{-4} \pm 4.85 \times 10^{-5}$	$3.42 \times 10^{-4} \pm 3.32 \times 10^{-5}$	1.08	0.27
Urea	3.79	$2.31 \times 10^{-3} \pm 1.35 \times 10^{-4}$	$2.25 \times 10^{-3} \pm 1.68 \times 10^{-4}$	1.03	0.48

The selected Nuclear Magnetic Resonance (NMR) peaks (chemical shifts in the second column) determined in the amniotic fluid ^{1}H-NMR spectra for each group, were used for the quantification of metabolites, reported as mean and relative standard deviation. Results were validated by the univariate t-test.

In a PCA model including DAI and CON group (n = 12), the largest variation in the data [60.2% of the explained variation (R^2X)] was related to daidzein supplementation (Fig. 4A). And the dietary daidzein factor shows clustering patterns or trends in the PCA model. An PLS-DA model (n = 12) is shown in Fig. 4B, the model clearly separated by the factor related to dietary, it was driven by daidzein supplementation in the first component [56.7% of the explained variation (R^2X)].

OPLS-DA model was used to investigate discrimination between dietary groups and to identify variables responsible for class separation. As is shown in Fig. 5A, a clear separation between the two groups of amniotic fluid samples is demonstrated by the score plot. The Q^2, which indicates the predictability of the model, was equal to 0.257, and the R^2, which indicates the fraction of explained variance of the variable, was 0.567. Cross-validation showed that the model was not over-fitted. Metabolites that contributed to the separation in OPLS-DA model are presented in Fig. 5B-C and Table 4. From the analysis, a significantly lower level of Hypoxanthine (8.21 ppm), Tyrosine (6.93 ppm), lactate (4.11 ppm), Fructose (4.10 ppm), and Fucose (4.09 ppm) was observed in Daidzein treated-group with respect to Control group. Moreover, the levels of Arginine (3.23 ppm), Creatine (3.04 ppm), and Citrate (2.69 ppm) was significantly increased upon daidzein treatment.

Table 4
List of differential metabolites in the amniotic fluid.

Metabolites	δ ^{1}H (ppm)[a]	Formula	VIP[b]	*P*-value
Hypoxanthine	8.21(s)	$C_5H_4N_4O$	1.396	0.015
Tyrosine	6.93(m)	$C_9H_{11}NO_3$	2.101	0.032
Lactate	4.11(q)	$C_3H_6O_3$	2.363	0.008
Fructose	4.10(m)	$C_6H_{12}O_6$	2.363	0.008
Fucose	4.09(dd)	$C_6H_{12}O_5$	2.400	0.008
Arginine	3.23(t)	$C_6H_{14}N_4O_2$	2.033	0.049
Creatine	3.04(s)	$C_4H_9N_3O_2$	1.701	0.027
Citrate	2.69(d)	$C_6H_8O_7$	1.706	0.047

[a] δ ^{1}H (ppm) corresponds to signals used for integration; s: singlet; d: doublet; t: triplet; dd: doublet of doublets; m: multiplet.

[b] Variable importance in the projection.

Metabolite pathway analysis

Starting from the quantitative evaluation of discriminating metabolites between DAI group and CON group, the Metabolic Pathway Analysis was performed to investigate on the potential pathways that may significantly impact upon biological process. According to both the *p*-value and the impact value, the analysis showed target pathways that could be potentially altered between DAI group and CON group (Fig. 6A). Results from the pathway analysis are shown in details in Supplementary Table 3, in which many pathways are tested at the same time with resulting statistical *p*-values, obtained for multiple testing. As shown in Fig. 6B and Fig. 7, D-Arginine and D-ornithine metabolism, Amino sugar and nucleotide sugar metabolism, Aminoacyl-tRNA biosynthesis, Arginine and proline metabolism, resulted in the most relevant metabolome views potentially involved in the observed variation of DAI and CON amniotic fluid metabolites, according to the *p*-value (-log(*p*)) and the impact value.

Discussion

Experimental and clinical studies have revealed that amniotic fluid plays a critial role in embryo development and growth. Amniotic fluid can serve as a permeable barrier environment for embryos, which is mainly composed of metabolites produced by fetal plasma through fetal skin. Importantly, amniotic fluid not only provides a mechanical barrier to the developing embryo, but also provides nutritional support and immune protection for embryos [30–32]. Previous study indicated that the composition of the amniotic fluid was affected by maternal nutrition. For instance, chitosan oligosaccharide was found to improve the antioxidant status and metabolic profiles of amniotic fluid, which subsequently improved the foetal survival and growth in a pig model [33]. Daidzein is a class of flavonoid compounds with estrogen properties and has been reported to have a potential effect on animal reproductive capacity [34–36]. In the present study, DAI supplementation significantly increased the number of viable embryos at 35 dg. The result is consistent with previous study that DAI supplementation significantly elevated the fetal growth at the early gestion stage in rats [19].

Previous study indicated that DAI can regulate the hypothalamic–pituitary–gonad axis of the endocrine system by competitively binding with estrogen receptors [17]. At the present study, we observed that daidzein supplementation significantly increased the levels of estrogen and tended to increase the activity of progesterone in the amniotic fluid, which is consistent with a previous study that daidzein could improve estrogen and progesterone levels of female rat during pregnancy [19]. These results suggested that estrogen and progesterone not only play an essential role in the implantation process, but also participate in the maintenance of pregnancy. Moreover, we found that DAI increased the IGF-I concentration in the amniotic fluid. IGF-I is one of the important growth factors that plays a crucial role in regulating development and growth [37]. Elevation of the estrogen and IGF-I levels may contribute to the improved fetal development and growth [19, 33].

It was reported that embryonic developmental injury could mediate the production of excessive TNF-α that serves as the promoter of the cytokine regulatory network, which triggers inflammatory response further [38]. In an *in vivo* study, it was shown that TNF-α elevated in amniotic fluid of women with preterm parturition [39]. Importantly, we found that supplementation with daidzein had a tendency to decrease the

pro-inflammatory cytokine TNF-α content of the amniotic fluid, which is consistent with a previous finding that daidzein supplementation suppressed pro-inflammatory cytokines such as TNF-α in the rat serum [40]. Thus, we speculated that daidzein supplementation can prevent embryonic developmental injury by decreasing the TNF-α level in the amniotic fluid. In mouse spleen cells treated with daidzein, an increase in the content of IgM in the culture supernatant was observed [41]. However, we found that daidzein supplementation tended to decrease the amniotic fluid concentrations of IgM compared to the CON group. This difference may be caused by different physiological states, and the specific mechanism needs further study.

Previous studies indicated that overproduction of oxygen radicals impair the embryo development [42]. Interestingly, the free radical-scavenging properties of daidzein has been demonstrated in several studies and reported to be more effective than that of the antioxidant vitamins in removing active oxygen [43–45]. Accordingly, we evaluated the antioxidant-related parameters of the amniotic fluid and found that daidzein supplementation increased the activities of GSH-Px in the amniotic fluid. However, daidzein supplementation had no significant influence on activities of SOD and T-AOC in the amniotic fluid. Although the activity of SOD and T-AOC has not changed, in a recent study, we noted that the GSH-Px act as a placental antioxidant enzyme that protects the vasculature from ROS, maintaining the normal pregnancy [46]. Based on the above novel findings, we concluded that daidzein plays an important role in enhancing the amniotic fluid antioxidant defense properties.

The metabolite analysis can provide comprehensive information about energy metabolism, precursors of proteins and carbohydrates, gene expression regulation, and signaling molecules [47]. Clinical studies have indicated that changes in amniotic fluid appear to be associated with preterm birth, preeclampsia, Down and Turner syndromes, and premature rupture of membranes (PROM) [48–50], which suggested that amniotic fluid metabolic profiles are potentially useful in the diagnosis of pregnancy disorders. In this study, we used ^{1}H NMR-based metabolomics approach to analyze amniotic fluid samples to identify the effect of daidzein supplementation on the endogenous metabolites.

The metabolites determined by ^{1}H-NMR in the present study responded differently to different dietary and significant differences in their levels were observed between the DAI group and CON group. The levels of Fucose, D-Fructose, and Lactate, which were related to the energy metabolism-related pathways, were significantly decreased in the DAI treatment group. Fucose and D-fructose, as substrates for energy metabolism, can be converted to pyruvate through the Amino sugar and nucleotide sugar metabolism as well as Starch and sucrose metabolism pathways, respectively. Lactate also represents an important substrate for energy metabolism. However, Lactate concentration can be used as a useful biomarker for predicting neonatal morbidity [51]. Interestingly, in the DAI group, a lower level of amniotic fluid Lactate was measured with respect to CON group. In addition, the level of Citrate, which as a fundamental metabolite for the tricarboxylic acid cycle [52], increased in the DAI-treated group compared to the control group. Based on our results, we speculate that an increased energy metabolisms levels may provide a strong scientific basis for pregnant supplemented with daidzein to improve embryo survival and growth.

We also found that the arginine and creatine levels in the DAI group were significantly increased compared to the CON group. Arginine and Creatine, which are related to the pathway of Arginine and proline metabolism, were converted and synthesized through the Citrate cycle. Arginine act as a precursor for the synthesis of nitric oxide (NO) that enhances placental blood flow during pregnancy [53], which corroborates the previous report that daidzein have been shown to increase NO release [54]. In addition, Arginine upregulates the gene expression of rapamycin signaling pathway targets, which coordinate anabolism and catabolism through multiple pathways to promote body growth [55]. Creatine is a metabolite involved in cellular energy homeostasis [56]. Creatine also has the potential to improve the survival and growth of embryos. Previous studies have reported that maternal dietary creatine supplementation during gestation could improve pregnancy outcomes [57]. Therefore, the improved embryo growth and development due to Daidzein supplementation occurs may as a result of changes in the concentrations of Arginine and Creatine.

Conclusions

In conclusion, our study provided the first evidence that daidzein supplementation during early pregnancy is beneficial for embryos growth and development. Such beneficial effects might be associated with an improvement immune and antioxidant status of amniotic fluid, as well as metabolic profiles, thereby creating an optimal internal environment for embryos growth. This study offers a molecular understanding of the potential mechanisms behind the improved embryos survival and development following daidzein supplementation, which may help to some extent transfer into the clinical arena in the future.

Abbreviations

DAI: Daidzein; ^{1}H–NMR: Nuclear magnetic resonance with respect to hydrogen-1 nuclei; E1: estrogen; IGF-I: insulin-like growth factor-I; TNF-α: tumor necrosis factor α; GSH-Px: glutathione peroxidase; EPL: Early pregnancy loss; VEGF: vascular endothelial growth factor; A4: androstenedione; T0: testosterone; E2: 17β-estradiol; P: progesterone; IgG: immunoglobulin G; IgA: immunoglobulin A; IgM: immunoglobulin M; IFN-γ: interferon γ; IL-1: interleukin-1; IL-6: interleukin-6; IL-10: interleukin-10; RT: room temperature; MDA: malondialdehyde; T-SOD: total superoxide dismutase; CAT: catalase; T-AOC: total antioxidative capability; TBARS: thiobarbituric acid reactive substance; TBA: thiobarbituric acid; SOD: Superoxyde dismutase; PQN: Probabilistic Quotient Normalization; PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; OPLS-DA: orthogonal partial least-squares discriminant analysis; VIP: variable importance in the projection; HCA: hierarchical cluster analysis; SD: standard deviations.

Declarations

Ethics approval and consent to participate

The experimental procedures followed the actual law of animal protection that were approved by the Animal Care Advisory Committee of Sichuan Agricultural University (No. 20190318) and were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Consent for publication

All the authors read and agree to the content of this paper and its publication.

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

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Authors' contributions

JH, JL, JY, PZ, XM, YL, ZH, HY, BY and DC participated in the design of the study. KX and YL collected the experiments data. KX analyzed the data and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Figures

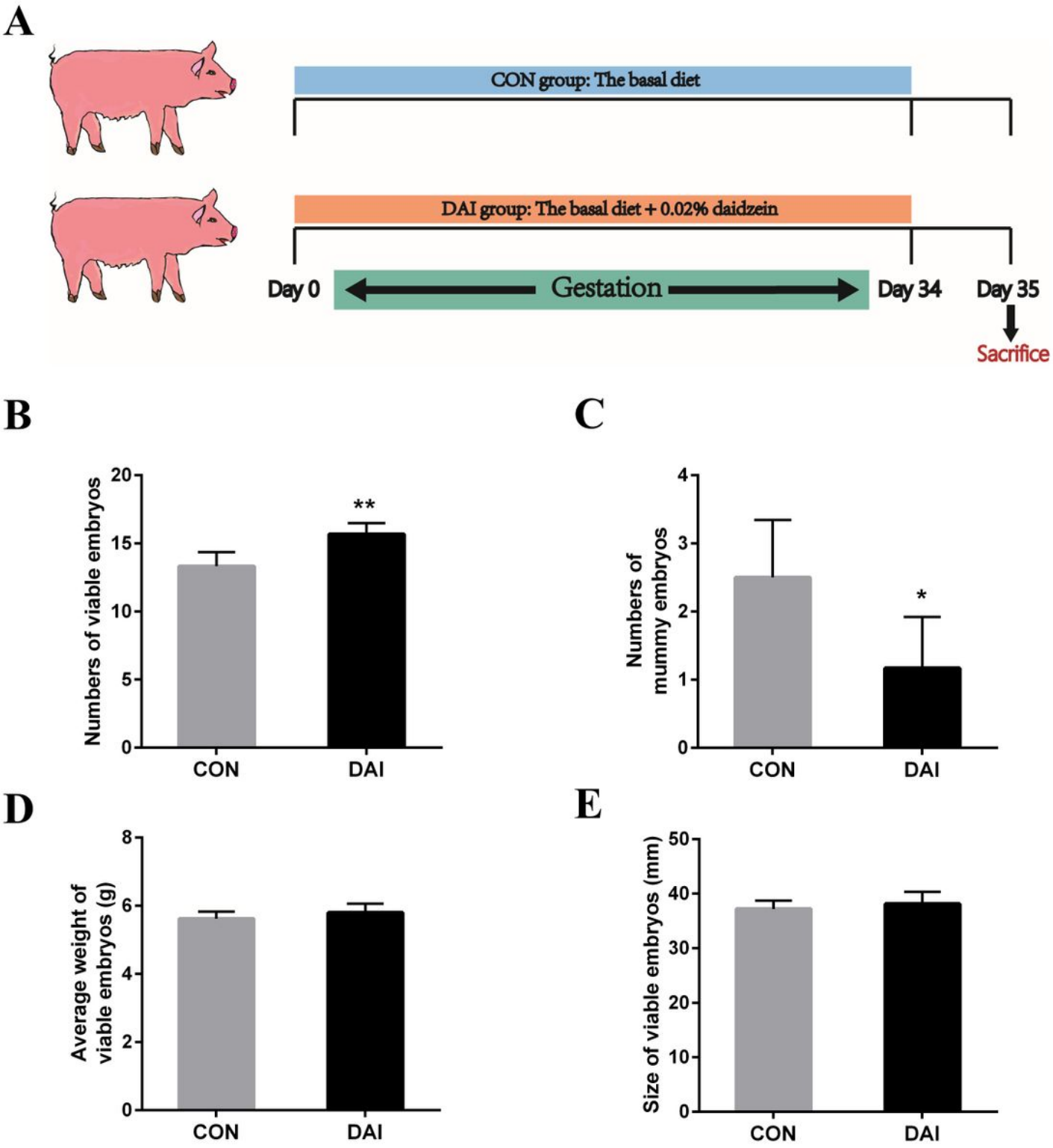

Figure 1

Effect of daidzein on the development of embryos at 35 days of gestation (dg). (A) Scheme of the animal experimental design. Sows were assigned to two groups randomly (DAI Group, n = 60; CON Group, n = 60). Sows were continuously fed with a basal diet or a basal diet plus an extra 0.02% daidzein for 35 days (day 0 to day 34). A random subset of sows (n = 6 per treatment) with close average body weight were sacrificed at day 35 of gestation. The numbers of viable (B) or mummy (C) embryos, and the

average weight (D) or size (E) of viable embryos are expressed as means with standard deviations (n=6); Size represents crown-to-rump length (mm) of viable embryos. DAI: A basal diet supplemented with 200 mg/kg daidzein; CON: A corn-soybean basal diet. *P < 0.05, **P < 0.01.

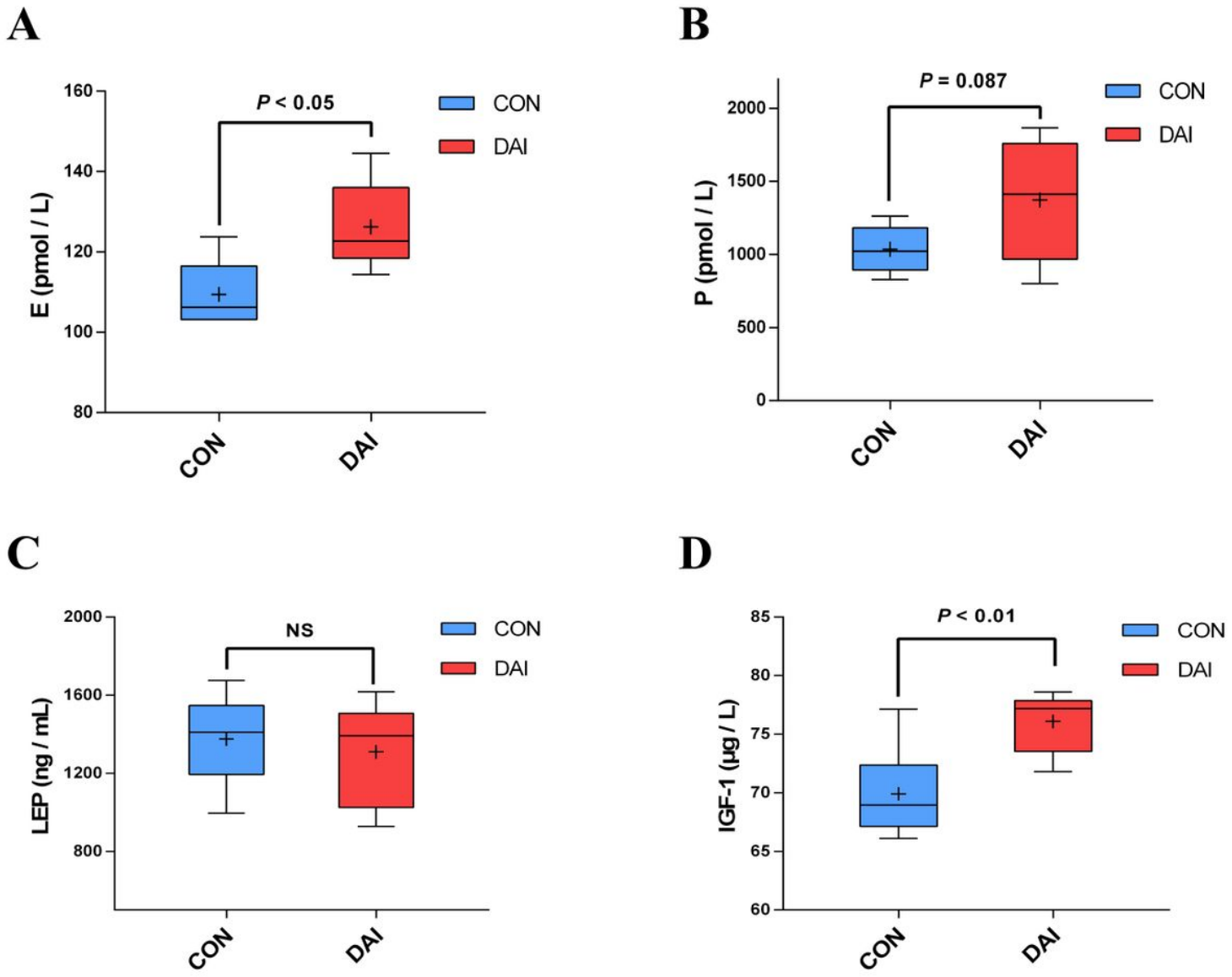

Figure 2

Effects of daidzein supplementation on the amniotic fluid reproductive hormone and growth-related factors levels. (A) E: Estrogen; (B) P: Progesterone; (C) LEP: Leptin; (D) IGF-1: Insulin-like growth factor-1. DAI: A basal diet supplemented with 200 mg/kg dietary daidzein; CON: A corn-soybean basal diet. Box and Whisker plots at 10-90th percentiles, including means with standard deviations (n=6).

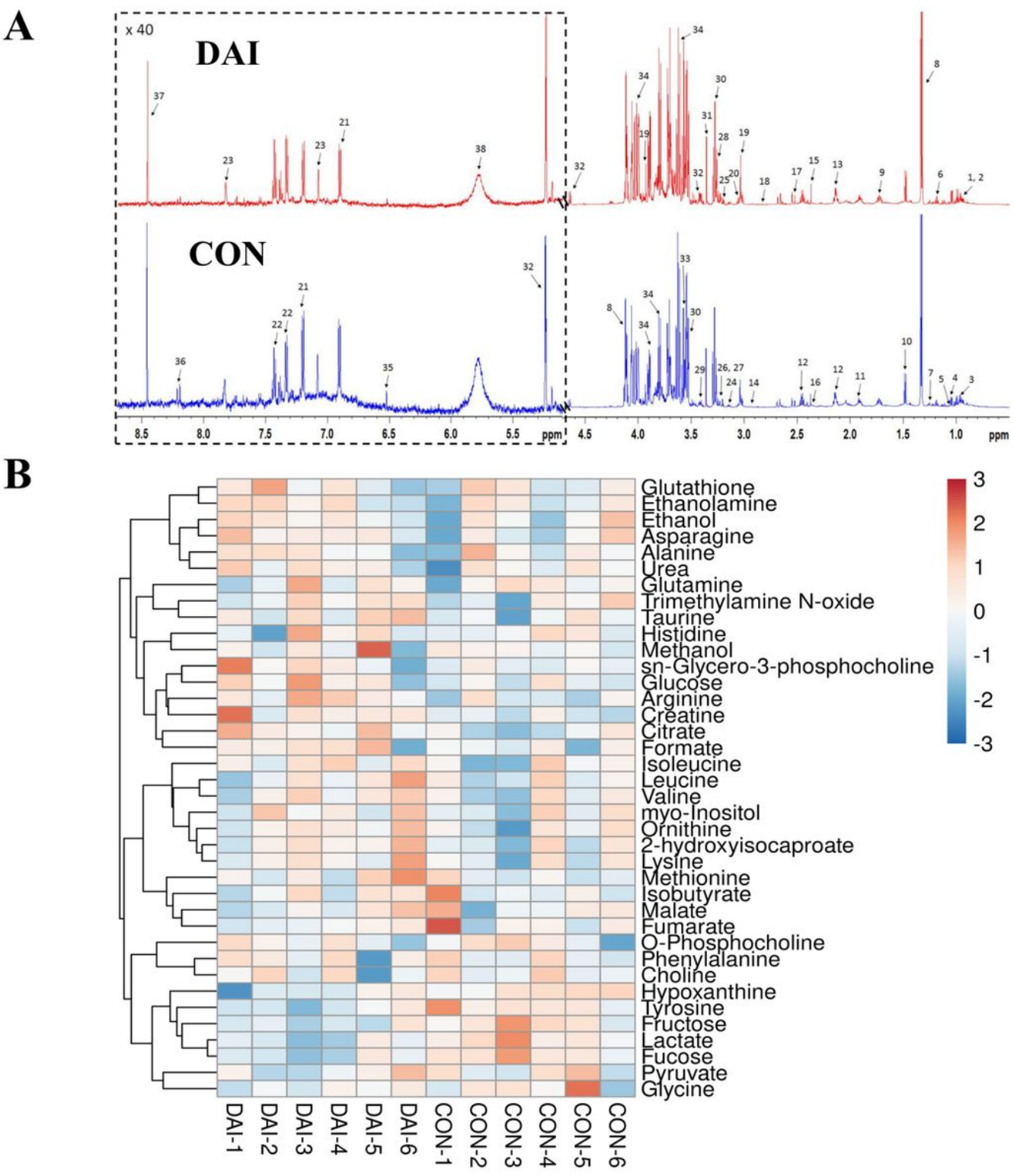

Figure 3

Identified metabolites with annotation in the amniotic fluid. (A) The representative 600 MHz 1H-NMR spectra of amniotic fluid samples. Key: 1, Isoleucine; 2, 2-hydroxyisocaproate; 3, Leucine; 4, Valine; 5, Isobutyrate; 6, Ethanol; 7, Fucose; 8, Lactate; 9, Lysine; 10, Alanine; 11, Arginine; 12, Glutamine; 13, Methionine; 14, Glutathione; 15, Pyruvate; 16, Malate; 17, Citrate; 18, Asparagine; 19, Creatine; 20, Ornithine; 21, Tyrosine; 22, Phenylalanine; 23, Histidine; 24, Ethanolamine; 25: Choline; 26, O-

Phosphocholine; 27, sn-Glycero-3-phosphocholine; 28, Trimethylamine N-oxide; 29, Taurine; 30, myo-Inositol; 31, Methanol; 32, Glucose; 33, Glycine; 34, Fructose; 35, Fumarate; 36, Hypoxanthine; 37, Formate; 38, Urea. (B) Heatmap of hierarchical clustering analysis (HCA) for identified metabolites of DAI group vs CON group amniotic fluid samples. The HCA based on the Euclidean distance; The colors from blue to red indicate the elevated content of metabolites. DAI: A basal diet supplemented with 200 mg/kg dietary daidzein; CON: A corn-soybean basal diet.

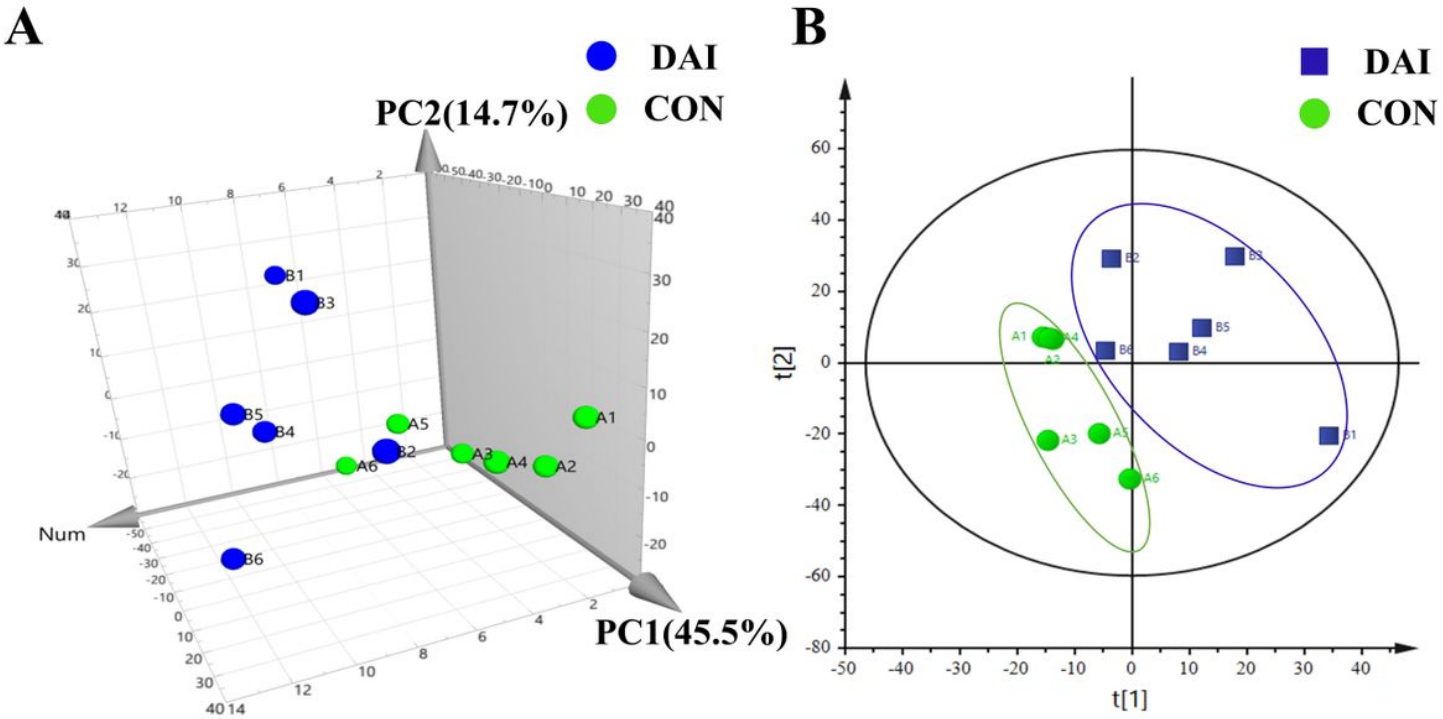

Figure 4

Score plots of 3D-PCA and PLS-DA separating DAI group from CON group. (A) The 3D-PCA plots (R2X= 60.2%, Q2= 0.384; A=5, N=12) and (B) PLS-DA scores plots (R2X=0.567, R2Y =0.75, Q2=0.36; A=1+1, N=12) derived from 1H-NMR spectra of amniotic fluid samples. CON: A corn-soybean basal diet; DAI: A basal diet supplemented with 200 mg/kg daidzein.

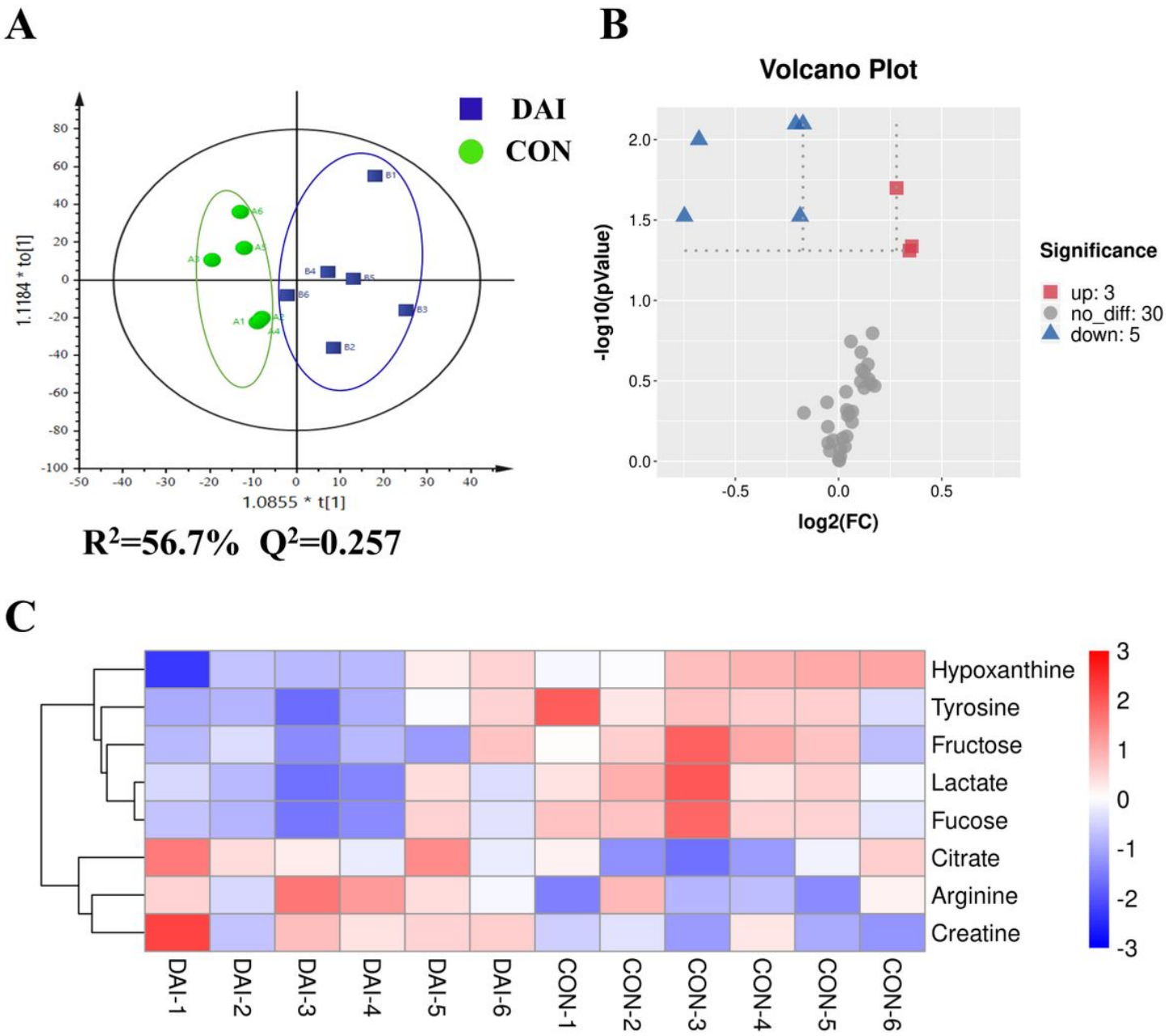

Figure 5

Identification of key discriminatory metabolites by the score plots of OPLS-DA. (A) OPLS-DA plots derived from 1H-NMR spectra of amniotic fluid samples (R2X= 56.7%, Q2= 0.357; A=1+1, N=12). (B) Volcano Plot representing the significant variables in the discrimination between DAI group and CON group. The significant increased variables are represented in red square; The significant increased variables are represented in blue triangle ($P < 0.05$; $FC > 1.1$). (C) Heatmap of hierarchical clustering analysis (HCA) for differential metabolites of DAI group vs CON group amniotic fluid samples. The HCA based on the Euclidean distance; The colors from blue to red indicate the elevated content of metabolites. CON: A corn-soybean basal diet; DAI: A basal diet supplemented with 200 mg/kg daidzein.

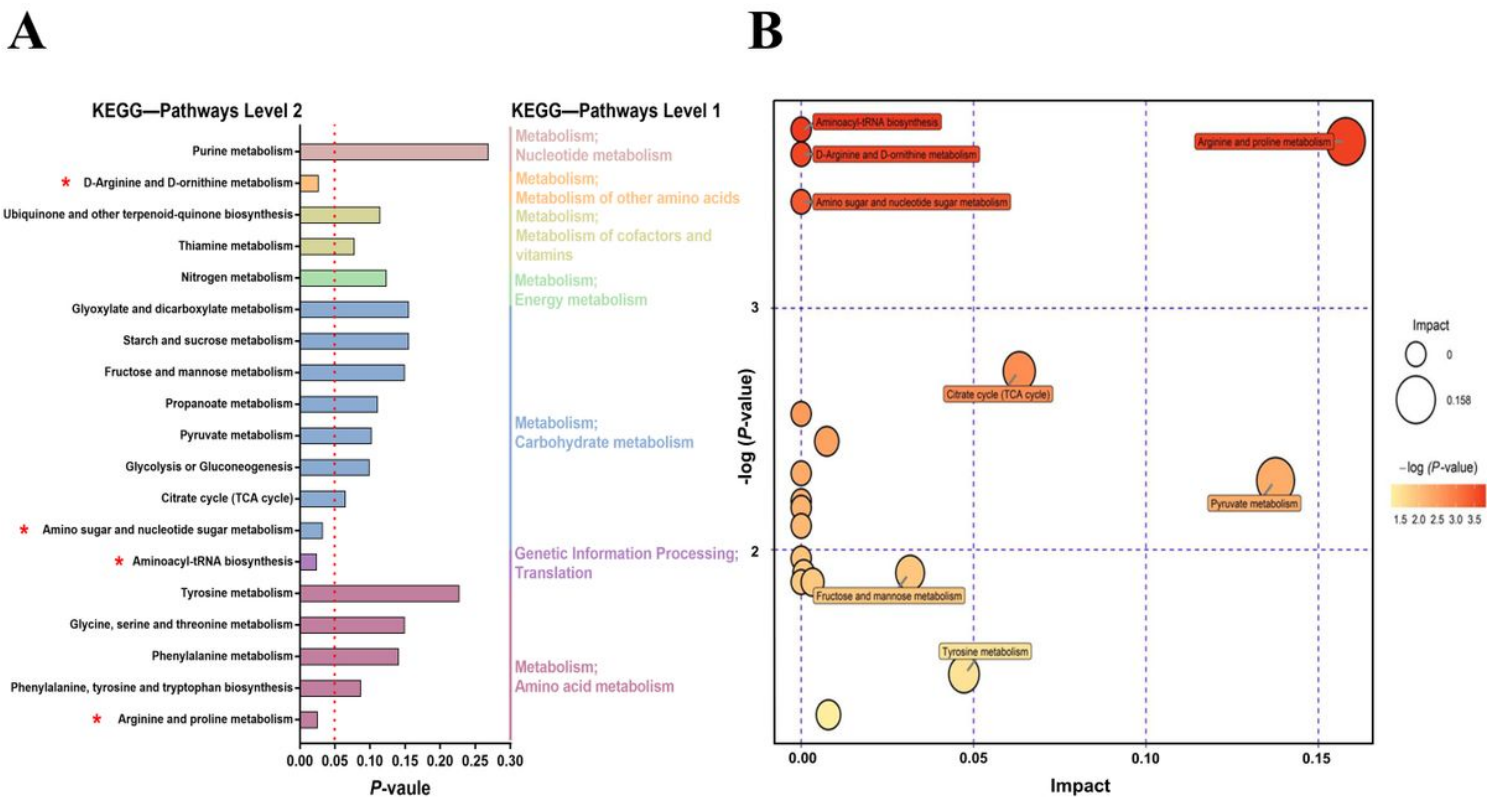

Figure 6

Relevant metabolic pathway in the comparison between DAI and CON group. (A) Clustering of KEGG pathways based on the key metabolites of amniotic fluid; (B) Bubble plot for identification of the most relevant metabolic pathways. Color of circles indicates the significance of changes of metabolites in the corresponding pathway while the size corresponds to the pathway impact score. The pathway impact score represents the cumulative percentage from the matched metabolite to the total pathway importance. *P < 0.05.

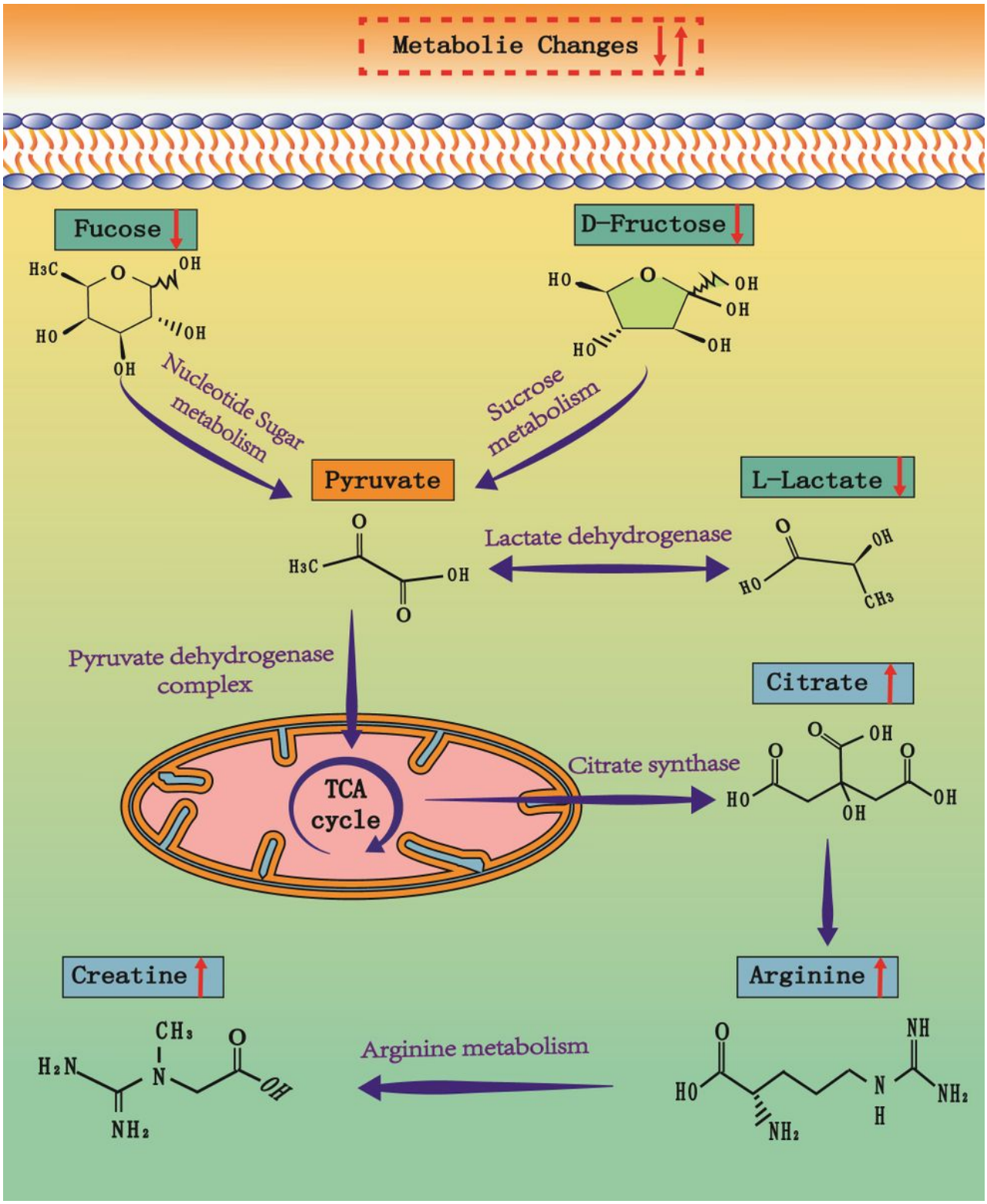

Figure 7

Schematic of the different biochemical pathways affected by Daidzein supplementation. Main pathways through which carbohydrates supply the Citrate cycle to furnish Arginine. Red arrows indicated the change direction: metabolite increased (upward arrow) and metabolite decreased (down arrow) respect to sows feed with a corn–soybean basal diet.

Supplementary Files

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- Supplementarymaterials.docx