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Dietary calcium and magnesium supplemented maternal diets on skewing of sex ratio and sexually dimorphic gene expression in Rabbit (*Oryctolagus cuniculus*) placenta

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ABSTRACT

This study aimed to investigate the influence of calcium and magnesium supplementation on the skewing of sex ratio and placental genes expression in New Zealand White Rabbits. A total of 25 rabbits were allocated to 5 groups; each treatment group was supplemented with Ca and Mg; T1 (0.4% and 0.01%), T2 (0.6% and 0.02%), T3 (0.8% and 0.03%), and T4 (1.0% and 0.04%) respectively, while the C group was provided with regular feed and subjected to three breeding. The T3 group produced a female-biased litter and hence, the F1 female kits (n=12) of T3 and C group was provided same supplementation management. At 21 days of gestation, three animals from each group were sacrificed and placental samples were collected, the remaining animals were allowed for full-term delivery. The selected F1 produced female-biased litter with elevated serum Ca and Mg concentrations and reduced sodium and cholesterol levels. A total of 15 genes related to mineral absorption, placental development and immunity were selected to study the influence of diet on sex and placental gene expression. The expression of genes such as *PEG10, SOD1, SLC30A, TLR4, AR,* and *TRPM6* was high in the treatment placenta compared to the control. *RTL1, ESR2, CALM2,* and *TRPM6* upregulated in the treatment female placenta. The study concluded that the intake of 0.8% Ca and 0.03% Mg could lead to the production of more females and upregulation of some placental genes could serve as the molecular mechanism in response to intake of the minerals and fetal sex.

Keywords: Calcium, Gene expression, Magnesium, Maternal diet, Placenta, Sex ratio

Pre-conceptual maternal diet influences the sex ratio (Rosenfeld *et al*. 2003, 2004, Cameron *et al*. 2008, Arangasamy *et al.* 2015). Animals supplemented with low-fat and high-fat diets skewed towards females and males, respectively (Rosenfeld *et al*. 2003). A maternal diet supplemented with minerals such as sodium-potassium skewed toward males while the diets supplemented with calcium-magnesium skewed toward females in rats and sheep (Arangasamy *et al.* 2015, Alhimaidi *et al*. 2021). These made maternal nutritional management and nutrient intake an explorable means for skewing of sex ratio.

The maternal diet during pregnancy can induce different physiological and gene expression changes in the fetus (Lan *et al*. 2013, Keleher *et al*. 2018). This is a process mediated by the placenta which is the major communicating organ between the mother and the developing fetus. It nourishes the growing embryo, facilitates the exchange of gases, nutrition, and waste products, and also mediates fetal programming (Jansson and Powell 2007). Despite sharing a common genome, males and females differ in terms of physiology and morphology, thus, there are sex-specific differences in placental gene expression (Mao *et al*. 2010). These developmental differences between the sexes initiated by the differential placental molecular function are critical for pre-and post-birth performance attributes. Since the placenta has the ability to survive and buffer against environmental insults, it is an appropriate organ to study the impact of maternal diet on the developing fetus. However, very few studies have investigated the effect of maternal diet on the pattern of placental gene expression (Gheorghe *et al*. 2009), especially in relation to sex type (Mao *et al*. 2010, Gabory *et al*. 2012). Therefore, in this present study, few functional expressions of genes associated with placental function, immune regulation, signal transduction, and fetal growth were investigated with respect to maternal diet. This study delineates the role of dietary compounds such as Ca and Mg on the biochemical parameters, sex ratio, and sex-specific placental gene expression.

MATERIALS AND METHODS

Animal management and breeding: The study was approved by the institutional animal ethics committee (IAEC) of ICAR-National Institute of Animal Nutrition

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and Physiology, Bengaluru (NIANP/IAEC/1/2019). The study was carried out in two generations; in the parental generation, 25 rabbits were randomly divided into 5 treatment groups each containing 5 animals (n=5). The control (C) group was provided with regular feed (VRK Nutritional Solutions, India) whereas graded amounts of Ca and Mg were mixed with the regular pellet feed for the treatment groups which include T1 (0.4% Ca and 0.01% Mg), T2 (0.6% Ca and 0.02% Mg), T3 (0.8% Ca and 0.03% Mg), and T4 (1.0% Ca and 0.04% Mg), respectively. The animals were placed in individual cages provided with 24 h drinking water and were provided with 16 h light and 8 h dark periods. The animals were subjected to three breeding of a 56-day reproductive rhythm. The T3 group produced a female-biased litter among all the three breeding. Hence, the female F1 kits (n=12) from the T3 group and the C group were selected and were continued with respective diets until maturity. The blood sample was collected from marginal ear vein before being serviced by active non-supplemented rabbit bucks. At 21 days of pregnancy when placental development could have been completed and the period when gonads of the fetus would have begun to develop (Daniel-Carlier *et al*. 2013), three rabbits from each group were sacrificed by administering sodium thiopentone anesthesia (50 mg/ kg). The kit sample and respective placenta sample were preserved separately. Later the DNA from kit tissue was isolated using the TRI reagent (Sigma Aldrich, USA) and the sexes were identified by employing the primers for *SRY* gene (Accession No. NM_001082253.1, F- 5' TACAGACCTCGTCGGAAGGT 3' and R- 5' TCTTGCCAGCTTGTCCAGTT 3' - 212bp) and *GAPDH* (Accession No. NM_001171148.1, F- 5' TGGAGAAAGCTGCTAAGTATG 3' and R- 5' CACAAAGTGGTCATTGAGGG 3' -179 bp) as house-keeping gene and the obtained PCR product was run on 2% agarose gel (Fig. 1).

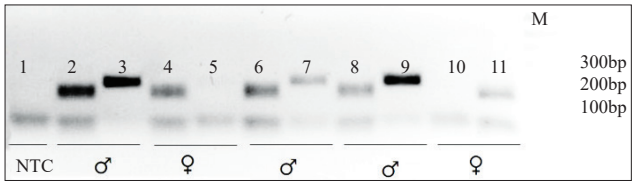

Fig. 1. Gender identification of the rabbit kits by PCR amplification of *SRY* gene. Lanes 2, 4, 6, 8 and 11 show an amplified product for the housekeeping gene (*GAPDH*), and lanes 3, 7 and 9 show an amplified product for *SRY* confirming the presence of male kits and lanes 5 and 10 having no amplified product confirms the presence of female kits. Lane 1 is a non-template control.

The rest of the animals were allowed for full-term delivery. The gender of the kits was identified by the anogenital sexing method (Nielsen and Torda 1983) within 24 h of kindling and again confirmed at 21 days of age.

Biochemical parameters estimation: Biochemical parameters were measured in serum collected before breeding. The glucose and cholesterol were estimated using the commercially available kit (Auto span, Arkray Healthcare (P) Ltd., India). Serum calcium was determined using the calcium kit (Arsenazo III, India). Serum magnesium was determined according to the manufacturer's instructions (Proton Biologicals India Pvt. Ltd., India). Serum electrolytes such as chloride and sodium were estimated using a commercially available kit (TRUEchemie, India) according to the manufacturer's instructions. The absorbance was measured using microplate spectrophotometer (Thermo Scientific multiskan FC microplate photometer, Finland).

RNA isolation and RT-qPCR: The placenta of each male and female kit (n=24) was used for gene expression analysis. RNA was isolated from placental tissue (30 mg) using trizol (Thermo Scientific, USA) as per the kit protocol.

The quality and quantity of RNA were checked using a spectrophotometer (Nanodrop 1000, Thermo Fisher, USA). Then the DNA-free kit (TURBO DNase, Ambion®, USA) was used to carry out DNase treatment as per the manufacturer's instructions. cDNA synthesis was carried out by using PrimeScript RT reagent (Takara, Japan).

qPCR was performed in Real-Time PCR (Step-one applied biosystems, USA) using SYBR Green Mastermix (TB Green Premix Ex Taq II, Takara, Japan). A total of 15 primers were designed using primer 3 software (NCBI) (Table 1). The protocol was set as: Initial denaturation at 95°C for 2 min, 95°C for 5 sec (40 cycles), 61°C for 10 sec, and extension for 15 sec at 72°C. *EIF4A2* was used as house-keeping gene. The relative gene expression was evaluated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Gene ontology: The differentially expressed genes were subjected to gene ontology functional enrichment analysis using ShinyGo (v0.75) by setting the best matching species as human and the P-value cut-off as 0.05.

Statistical analysis: The significance of biochemical parameters, sex ratio, and gene expression were analyzed by student's t-test. Data were expressed as mean±SEM, and the values were considered to be significant at $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

In this study, the effect of maternal diet was assessed on sex ratio, expression of genes associated with placental function, immune regulation, signal transduction, mineral absorption and fetal growth. The findings suggest that a particular sex type might be associated with the ability of the placentae to survive and buffer against fetal environmental insults. The observed lower serum concentration of Na, higher serum concentration of Ca and Mg levels in the animals maintained on the Ca and Mg diet (Table 2) are consistent with the earlier studies (Noorlander *et al.* 2010, Alhimaidi *et al*. 2021). The intake of the Ca and Mg-enriched diet has cholesterol-lowering properties (Vaskonen *et al*. 2002), and a reduction in cholesterol levels in the treatment group was observed (Table 2).

Furthermore, the findings in the present study are

Table 1. List of primers used for gene expression studies

Gene	Primer sequence (5' – 3')	Product length	Accession No.
ABCA1	FP- TGAAAAAGGTGGTGCCTGAG RP- TACGGCAGCACATAGGTCAG	181	XM_008257418.2
OGT	FP- AGCACAGAACCAACGAAACG RP- CAGCCTTCGACACTGGAAGT	183	XM_002720103.3
PEG10	FP- AACCCCTGCAACAGGAAACA RP- GCGTCATTGAGAGGTGGTCA	221	NM_001302483.1
RTL1	FP- GAGGGCAACTTCATGGACGA RP- GAGAAGTCGGGCGAAGGTAG	203	XM_008252457.1
TRPM6	FP- GAAACTCCGGAGGAACCTGG RP- CTTTCACCTGAGGACGCAGT	245	XM_008256490.2
AR	FP- GGTGAGCAGAGTGCCCTATC RP- ACCTTGCAGCTTCCACAAGT	222	NM_001195724.1
ATP2A1	FP- AAAGTCCCTGCAGACATCCG RP- GCGATGTTGGTACCCGAGAA	173	NM_001089318.1
ESR2	RP- CTTGCAGGAAGTGGACCCAT FP- GGTGGGCAGTGACGATAACA	159	XM_017349392.1
IL-8	FP- AAGTGGGTGCAGAAGGTTGT RP- GCCCTACGACAGATCCATGC	166	NM_001082293.1
MAP2K1	FP- CTACAGCGATGGCGAGATCA RP- AGGATGTTGGAGGGCTTCAC	192	NM_001082629.1
TLR4	FP- TGTGTGGAGGTCGTTCCCAATA RP- AGGCCTTGGTATGCATCATCT	218	NM_001082732.2
TRPV6	FP- GGATGAGCTGGGCCATTTCT RP- CAGTGAGTGTCGCCCATCAT	198	NM_001082776.1
SLC30A7	FP- GGCAAAGAAGATGTTGCCCC RP- AGTTGCTCCAGATGCCGTAG	191	XM_008264812.2
SOD1	FP- GGTGGTCAAGGGACGCATAA RP- CACATCAGCCACACCATTGC	205	NM_001082627.2
CALM2	FP- TCTCTTGGGCAGAATCCCAC RP- TTGTCATCACATGGCGAAGC	220	NM_001195640.1
EIF4A2	FP- CGATGGTGTCATCGAGAGCAA RP- GTGGCTGTCTTGCCAGTACC	198	XM_002716453.3

Table 2. Effect of Ca and Mg supplemented diet on biochemical parameters in rabbits

Group	Glucose	Cholesterol	Calcium	Magnesium	Sodium	Chloride
Control	120.12±5.01	68.76±4.31*	11.44±0.14*	2.55±0.06*	140.81±2.22*	98.77±1.31
Treatment	109.12±4.87	57.20±3.03*	11.95±0.15*	3.14±0.25*	135.54±1.80*	99.68±1.61

Data is presented as Mean ± SE. The significant difference was determined at * $p < 0.05$ compared to control.

Table 3. Effect of Ca and Mg supplemented diet on sex skewing in rabbits

Group	Total number of kits	Number of male kits	Number of female kits	Male kits (%)	Female kits (%)	Sex ratio (Female: Male)
Control	81	41	40	48.97	51.03	0.98
Treatment	86	32	54	37.12*	62.88*	1.69

Significant difference was determined at * $p < 0.05$ compared to control.

consistent with the previous studies on the supplementation of Ca and Mg in maternal diets resulting in the production of the female-biased sex ratio (Table 3) in rats and sheep supplemented with different levels of Ca and Mg (Abd Elraouf Oun *et al*. 2016, Alhimaidi *et al*. 2021). A study by Gharibi and colleagues (2023) investigated the effects of dietary Ca and Mg supplementation on the sex ratio of lambs. The study found that ewes supplemented with higher levels of Ca and Mg gave birth to a higher proportion of female lambs. The mechanism underlying the effect of Ca and Mg on the sex ratio of offspring is not fully understood. However, it has been suggested that Ca and Mg may influence the production and function of hormones involved in fetal development, such as estrogen and progesterone. In attestation to this, a study by Hassan *et al*. (2022), showed that increasing the Ca:P ratio in the maternal diet resulted in a sex-specific morphological changes and mineral accretion in female offspring.

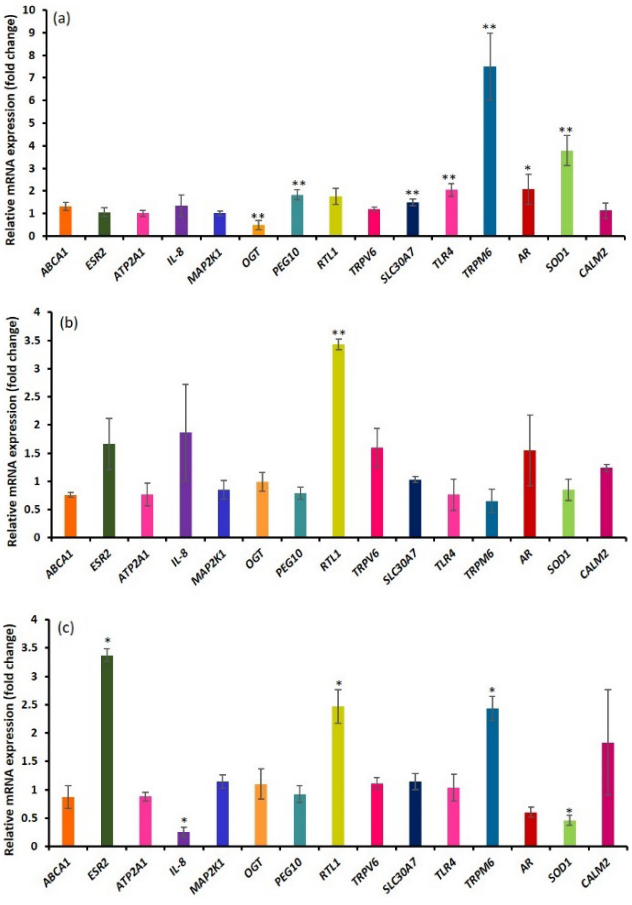

Fig. 2. The relative placental mRNA expression levels of *ABCA1, ESR2, ATP2A1, IL-8, MAP2K1, OGT, PEG10, RTL1, TRPV6, SLC30A7, TLR4, TRPM6, AR, SOD1,* and *CALM2* in Ca and Mg treated animals by RT-qPCR. The changes in CT values were normalized with the housekeeping gene (*EIF4A2*). Fold change in control is considered as 1. The relative mRNA expression levels in the treatment group compared to control (a) Change in relative mRNA expression of control female placenta compared to the control male placenta; (b) Change in relative mRNA expression in treatment female placenta compared to treatment male placenta; (c) Significant difference at ** $p < 0.01$, * $p < 0.05$ when compared to control group.

The placental genes expression was compared between F1 control and treatment group animals (Fig. 2). The expression of genes such as *PEG10* (1.66 fold), *SOD1* (3.26 fold), *SLC30A7* (1.97 fold), *TLR4* (1.88 fold), *AR* (2.73 fold), and *TRPM6* (3.49 fold) in the treatment group was significantly ($p < 0.01$) high compared to the control indicating molecular implication of the intake of the mineral towards the production of more female. The expression of the *OGT* (0.57 fold) gene was significantly low compared to the control. The gene ontology functional enrichment analysis of the upregulated genes was involved in biological processes like regulation of cellular response to oxidative stress (*TLR4* and *SOD1*; enrichment FDR 0.0175), B cell proliferation involved in immune response (*TLR4*; enrichment FDR 0.0191), response to endogenous stimulus (*TLR4, SOD1, AR,* and *PEG10*; enrichment FDR 0.0175), organ growth (*SOD1* and *AR*; enrichment FDR 0.0180), epithelial cell development (*SOD1* and *AR*; enrichment FDR 0.0191), and plasma membrane organization (*SOD1* and *AR*; enrichment FDR 0.0175). The B cells are part of the maternal immune system for immune regulation during pregnancy, which is an indication that the production of more females over males could be related to the maternal immune status (Li *et al*. 2020). This present study also showed that the placenta may be associated with the response to an endogenous stimulus like changes in nutrients, salts and secretion, as well as the regulation of cellular response to oxidative stress is important to protect the fetus from ROS (Takehara *et al.* 1990).

The molecular function of these upregulated genes also includes pattern recognition receptor activity (*TLR4*; enrichment FDR 0.0433), androgen binding (*AR*; enrichment FDR 0.0217), zinc ion binding (*SOD1, AR,* and *PEG10*; enrichment FDR 0.0217), superoxide dismutase activity (*SOD1*; enrichment FDR 0.0217), and transition metal ion binding (*SOD1, AR,* and *PEG10*; enrichment FDR 0.0217) (Fig. 3). Pattern recognition receptor activity is an important characteristic of the innate immune system to protect against infection (Hoo *et al.* 2020). Antioxidant activity in the placenta increases as the gestation progresses to combat the production of ROS due to high metabolic activity (Jones *et al.* 2010, Takehara *et al.* 1990). Metal ion binding activity in the placenta is required for detoxification to protect the fetus (Chow *et al*. 1987). Androgens play an important role in placental development, implantation, and maintenance of pregnancy (Parsons and Bouma 2021).

Furthermore, to check the difference in placental gene expression between the sexes, we compared control males and control females as well as treatment males and females. The *RTL1* gene was observed to be significantly higher (3.43 fold; $p<0.05$) in the control female placenta compared to the control male placenta. *RTL1* is vital for placental development, and the deletion of which causes growth retardation of the placenta and the fetus (Sekita *et al*. 2008). Also, compared with the treatment of male and female placenta, the expression of *RTL1* (2.47 fold), *ESR2* (3.37 fold), *CALM2* (1.84), and *TRPM6* (2.44 fold) were upregulated; *IL-8* (0.26 fold) and *SOD1* (0.46 fold) were significantly low ($p<0.05$), in the treatment female placenta. The upregulated genes were involved in molecular functions such as steroid hormone receptor activity (*ESR2*; enrichment FDR 0.0423), kinase activity (*TRPM6* and *CALM2*; enrichment FDR 0.0423), and are involved in pathways such as mineral absorption (*TRPM6*; enrichment FDR 0.0402), estrogen signaling pathway (*ESR2* and *CALM2*; enrichment FDR 0.0106), and endocrine resistance (*ESR2*; enrichment FDR 0.0402) (Fig 3). *TRPM6* is essential for embryonic development as it is involved in maternal-fetal Mg transport and regulates Mg balance (de Clercq *et al.* 2021). It is also responsible for mother-fetus Ca transport to meet the demand for fetal bone mineralization (Suzuki *et al*. 2008). *ESR2* is involved in cell signaling needed for placental development (Uniprot). IL-8 is a proinflammatory cytokine

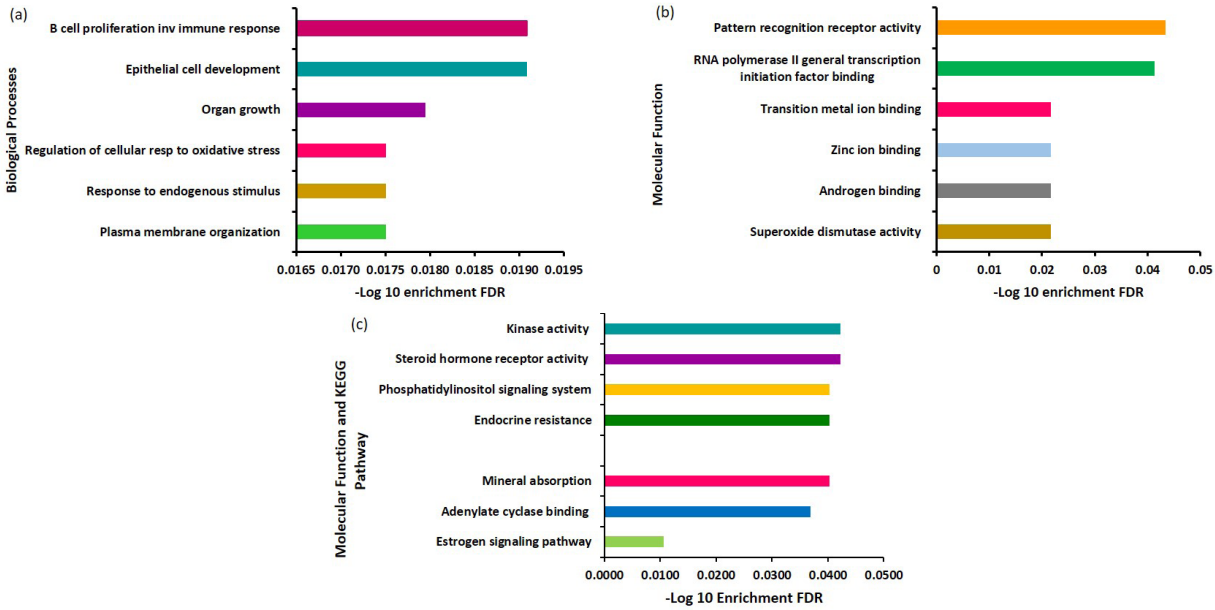

Fig. 3. Gene ontological functions and pathways enriched in the placenta of rabbits. (a) Biological processes enriched in treatment group placenta; (b) Molecular functions enriched in treatment group placenta; (c) Molecular functions and KEGG pathways enriched in treatment female placenta.

responsible for neutrophil infiltration to the site of injury (Harada *et al*. 1994). The expression of proinflammatory mediators is more in the male placenta may be because of less maternal-fetal compatibility compared to that of females (Cvitic *et al*. 2013). The antioxidant gene expression *SOD1* was observed to be high in the male placenta. The antioxidant enzymes are important to combat the production of ROS due to metabolic activity during fetal growth. The male embryos grow faster compared to females (Mittwoch 1993) and hence may produce more ROS due to high metabolic activity.

This study highlights the potential benefits of supplementing diets with Ca and Mg in skewing the sex ratio towards female offspring. The results suggest that changes in placental gene expression, influenced by maternal diet and offspring sex, are associated with this effect. Furthermore, the study observed variations in serum biochemical profiles, including increased Ca and Mg levels and reduced sodium and cholesterol, in the treatment group. The placental gene expression in this group showed upregulation of genes such as *PEG10, SOD1, SLC30A, TLR4, AR* and *TRPM6,* which are involved in various biological processes such as regulating cellular response to oxidative stress, immune response, organ growth, and epithelial cell development. The upregulated genes also had molecular functions such as pattern recognition receptor activity, androgen binding, metal ion binding, and superoxide dismutase activity. Interestingly, the control female placenta exhibited higher expression of the *RTL1* gene compared to males. The study also observed upregulation of genes such as *RTL1, ESR2, CALM2* and *TRPM6* in the female placenta of the treatment group, which are involved in signaling and mineral absorption. However, further studies are needed to fully understand the molecular mechanisms involved in the skewing of sex ratio and placental gene expression. Overall, the findings suggest that Ca and Mg supplementation in maternal diets can influence the sex ratio of offspring by modulating placental gene expression and altering serum biochemical profiles.

REFERENCES

Abd Elraouf Oun S B, Soltan S, Taha A and Kadry E. 2016. Preconceptional minerals administration skewed sex ratio in rat offspring. *Research Journal of Obstetrics and Gynecology* **7**: 11–15.

Alhimaidi A R, Ammari A A, Alghadi M Q, al Saiady M Y, Amran R A and Swelum A A. 2021. Sex preselection of sheep embryo by altering the minerals of maternal nutrition. *Saudi Journal of Biological Sciences* **28**(1): 680–4.

Arangasamy A, Selvaraju S, Parthipan S, Somashekar L, Rajendran D and Ravindra J P. 2015. Role of calcium and magnesium administration on sex ratio skewing, follicular fluid protein profiles and steroid hormone level and oocyte transcripts expression pattern in Wistar rat. *Indian Journal of Animal Sciences* **85**(11): 1190–4.

Cameron E Z, Lemons P R, Bateman P W and Bennett N C. 2008. Experimental alteration of litter sex ratios in a mammal. *Proceedings of the Royal Society B: Biological Sciences* **275**(1632): 323–7.

Chow S N, Chien C H, Chen Y P and Ouyang P C. 1987. Purification and characterization of metal-binding proteins and the corresponding mRNA from human placentas. *Biological Research in Pregnancy and Perinatology* **8**(2 2D Half): 65–72.

Cvitic S, Longtine M S, Hackl H, Wagner K, Nelson M D, Desoye G and Hiden U. 2013. The human placental sexome differs between trophoblast epithelium and villous vessel endothelium. *PLoS ONE* **8**(10): e79233.

Daniel-Carlier N, Harscoët E, Thépot D, Auguste A, Pailhoux E and Jolivet G. 2013. Gonad differentiation in the Rabbit: Evidence of species-specific features. *PLoS ONE* **8**(4).

de Clercq K, Pérez-García V, van Bree R, Pollastro F, Peeraer K, Voets T and Vriens J. 2021. Mapping the expression of transient receptor potential channels across murine placental development. *Cellular and Molecular Life Sciences* **78**(11): 4993.

Gabory A, Ferry L, Fajardy I, Jouneau L, Gothié J D, Vigé A, Fleur C, Mayeur S, Gallou-Kabani C, Gross M S, Attig L, Vambergue A, Lesage J, Reusens B, Vieau D, Remacle C, Jais J P and Junien C. 2012. Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta. *PLoS ONE* **7**(11): 47986.

Gharibi Z, Shamsolahi M, Fatahnia F, Mohammadi Y and Shokri A N. 2023. Effect of calcium and magnesium supplementation of ewes during pre-and post-mating on lamb sex ratio. *Iranian Journal of Applied Animal Science* **13**(1): 67–75.

Gheorghe C P, Goyal R, Holweger J D and Longo L D. 2009. Placental gene expression responses to maternal protein restriction in the mouse. *Placenta* **30**(5): 411–7.

Harada A., Sekido N, Akahoshi T, Wada T, Mukaida N and Matsushima K. 1994. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *Journal of Leukocyte Biology* **56**(5): 559–64.

Hassan M G, Chen C, Ismail H A, Zaher A R, Cox T C, Goodwin A F and Jheon A H. 2022. Altering calcium and phosphorus supplementation in pregnancy and lactation affects offspring craniofacial morphology in a sex-specific pattern. *American journal of orthodontics and dentofacial orthopedics: Official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics* **161**(5): e446.

Hoo R, Nakimuli A and Vento-Tormo R. 2020. Innate immune mechanisms to protect against infection at the human decidual-placental interface. *Frontiers in Immunology* **11**.

Jansson T and Powell T L. 2007. Role of the placenta in fetal programming: Underlying mechanisms and potential interventional approaches. *Clinical Science* **113**(1): 1–13.

Jones M L, Mark P J, Lewis J L, Mori T A, Keelan J A and Waddell B J. 2010. Antioxidant defenses in the rat placenta in late gestation: Increased labyrinthine expression of Superoxide Dismutases, Glutathione Peroxidase 3, and Uncoupling Protein 2. *Biology of Reproduction* **83**(2): 254–60.

Keleher M R, Zaidi R, Shah S, Oakley M E, Pavlatos C, Idrissi S, Xing X, Li D, Wang T and Cheverud J M. 2018. Maternal high-fat diet associated with altered gene expression, DNA methylation, and obesity risk in mouse offspring. *PLoS ONE* **13**(2): e0192606.

Lan X, Cretney E C, Kropp J, Khateeb K, Berg M A, Peñagaricano F, Magness R, Radunz A E, Khatib H, Dovc P and Liu H C. 2013. Maternal diet during pregnancy induces gene expression and dna methylation changes in fetal tissues in sheep. *Frontiers in Genetics* **4**: 49.

Li X, Zhou J, Fang M and Yu B. 2020. Pregnancy immune tolerance at the maternal-fetal interface. *International Reviews of Immunology* **39**(6): 247–63.

Livak K J and Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative pcr and the 2 C T method. *Methods* **25**: 402–8.

Mao J, Zhang X, Sieli P T, Falduto M T, Torres K E and Rosenfeld C S. 2010. Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. *Proceedings of the National Academy of Sciences of the United States of America* **107**(12): 5557.

Mittwoch U. 1993. Blastocysts prepare for the race to be male. *Human Reproduction (Oxford, England)* **8**(10): 1550–5.

Nielsen H C and Torda JS. 1983. Anatomy of fetal rabbit gonads and the sexing of fetal rabbits. *In Laboratory Animals* **17**.

Noorlander A M, Geraedts J P M, and Melissen J B M. 2010. Female gender pre-selection by maternal diet in combination with timing of sexual intercourse - A prospective study. *Reproductive BioMedicine Online* **21**(6): 794–802.

Parsons A M and Bouma G J. 2021. A potential role and contribution of androgens in placental development and pregnancy. *Life* **11**(7).

Rosenfeld C S and Roberts RM. 2004. Maternal diet and other factors affecting offspring sex ratio: A review. *Biology of Reproduction* **71**(4):1063–70.

Rosenfeld C S, Grimm K M, Livingston K A, Brokman A M, Lamberson W E and Roberts R M. 2003. Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. *Proceedings of the National Academy of Sciences of the United States of America* **100**(8): 4628–32.

Sekita Y, Wagatsuma H, Nakamura K, Ono R, Kagami M, Wakisaka N, Hino T, Suzuki-Migishima R, Kohda T, Ogura A, Ogata T, Yokoyama M, Kaneko-Ishino T and Ishino F. 2008. Role of retrotransposon-derived imprinted gene, Rtl1, in the feto-maternal interface of mouse placenta. *Nature Genetics* **40**(2): 243–8.

Suzuki Y, Kovacs C S, Takanaga H, Peng J, Landowski C P and Hediger M A. 2008. Calcium channel TRPV6 is involved in murine maternal–fetal calcium transport. *Journal of Bone and Mineral Research* **23**(8): 1249.

Takehara Y, Yoshioka T and Sasaki J. 1990. Changes in the levels of lipoperoxide and antioxidant factors in human placenta during gestation. *Acta Medica Okayama* **44**(2): 103–11.

Vaskonen T, Mervaala E, Sumuvuori V, Seppänen-Laakso T and Karppanen H. 2002. Effects of calcium and plant sterols on serum lipids in obese Zucker rats on a low-fat diet. *British Journal of Nutrition* **87**(3): 239–45.