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Molecular insights on skewing of sex ratio in rabbits (*Oryctolagus cuniculus*) supplemented with dietary calcium and magnesium

Sharanya Jeevendra Naidu[1,2], Arangasamy Arunachalam[1]*, Akeem Babatunde Sikiru[1,3], Selvaraju Sellappan[1,4], Backialakshmi Sekar[1], Ippala Janardhan Reddy[5], Raghavendra Bhatta[6]

[1] Animal Physiology Division, Reproductive Physiology Laboratory, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India; [2] Department of Biochemistry, Jain University, Bengaluru, India; [3] Department of Animal Production, Federal University of Technology, Minna, Nigeria; [4] ICAR - National Fellow, Animal Physiology Division, Reproductive Physiology Laboratory, Bengaluru, India; [5] Animal Physiology Division, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India; [6] Director, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India.

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Abstract

The effect of dietary calcium (Ca) and magnesium (Mg) supplementation on serum biochemical parameters, steroid hormones, gene expression, and the sex ratio was investigated in female New Zealand white rabbits. A total of 25 rabbits were allocated into five treatment groups: The control group was fed with regular pellet feed, whereas, treatment groups were supplemented with Ca and Mg: T1 (0.40% and 0.01%), T2 (0.60% and 0.02%), T3 (0.80% and 0.03%) and T4 (1.00% and 0.04%), respectively. The rabbits were subjected to three breeding cycles. The T3 group skewed towards females (65.33%) from all three breeding. There was elevated Ca concentration in T3 (15.26 ± 0.77 mg dL^{-1}) and T4 (15.61 ± 0.82 mg dL^{-1}) groups compared to the control. The concentration of estradiol was significantly high in T3 and T4 groups at 0.5 days post-coitus (dpc) and T2, T3 and T4 groups at 21dpc. Testosterone was significantly high in T4 group at 0.50 dpc and T2 and T4 group at 21dpc. The expression of 13 genes was studied in the oviduct. Genes such as *OVGP1, CCT4, ANXA2* and *TLR4* were up-regulated and positively correlated with the female sex ratio. The molecular functions and pathways of up-regulated genes were suggestive of their role in fertilization such as sperm selection, sperm storage, immune regulation, implantation and early embryonic development. The variations in the serum electrolytes, steroid hormones and gene expression might have an impact on the skewing process.

Introduction

The sex ratio is a key factor associated with productivity and profitability in the livestock industries because obtaining offspring of desired sex adds economic value to the livestock enterprise. Several methods have been applied so far at paternal and maternal levels to obtain offspring of the desired sex type. Technique such as flow cytometry being employed for the separation of the spermatozoa is not economic.[1]

Maternal diet and sex ratio alteration has received great interest over the decades.[2,3] In addition to these, there are several supporting evidence of maternal influence on the sex of the offspring. Investigations showed that a fat-rich maternal diet increased the sex ratio towards males in mice[4,5] and ewes.[6,7] Steroid hormone variation in the circulation and low vaginal pH among the fat-fed groups were identified to favor the motility of a selective population of spermatozoa.[8]

Besides, increasing intakes of certain minerals in the maternal diet around the time of conception was reported to influence the sex ratio of the offspring.[9,10] Supplementation of maternal diet with calcium (Ca) and magnesium (Mg) before mating skewed sex of the offspring towards females in rats and sheep[9,11] while increased sodium (Na) and potassium (K) diets skewed towards males in rats and sheep.[10,11] Females with high levels of testosterone and glucose produced more males in Field Voles,[12] Ibexes,[13] Macaques,[14] and low estradiol levels were found in females which produced a male-biased litter in grey mouse lemur.[15] The change in maternal nutrition probably modifies the oviduct environment or circulating steroid levels for favoring one population of spermatozoa over the other to move faster or reach the site of

***Correspondence:**

Arangasamy Arunachalam. MVSc, PhD, Post Doc.
Animal Physiology Division, Reproductive Physiology Laboratory, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India
E-mail: a.arangasamy@icar.gov.in

fertilization. The oviduct is the site where fertilization takes place.[16] The oviduct may recognize the X- and Y-sperm and modulate the environment for successful fertilization by eliciting differential transcriptomic responses.[17] Maternal diet may have an impact on the female reproductive system, hence, exploring the mechanisms at the molecular level is necessary.

So far studies on mineral influence on sex ratio were investigated, however, there is inadequate evidence on the influence of different levels of the combination of Ca and Mg supplementation on the biochemical parameters, hormone levels, gene expression, pregnancy features and sex ratio. This study was the first of its kind to examine the gene expression in the oviduct related to mineral intake and the skewing process. Therefore, the present study aimed to explore the mechanisms underlying the sex pre-selection leading to the production of more female offspring via increasing the maternal intakes of Ca and Mg.

Materials and Methods

Animals and experimental design. The study was carried out using 25 female New Zealand White rabbits of eight months of age with bodyweight ranging from 2.35 - 2.80 kg. The rabbits were allocated into five treatment groups: The control group was fed with regular pellet feed (VRK Nutritional Solutions, Maharashtra, India), whereas, treatment groups were supplemented with Ca and Mg: T1 (0.40% and 0.01%), T2 (0.60% and 0.02%), T3 (0.80% and 0.03%) and T4 (1.00% and 0.04%), respectively. The proximate composition of the feed comprises of dry matter = 91.48%, total ash = 8.64%, crude fibre = 11.16%, crude fat = 1.84%, crude protein = 17.80%, metabolizable energy = 2,700 kcal kg^{-1}, and acid in soluble ash = 1.77%. The mineral composition of the feed provided for control (C) and treatment groups supplemented with Ca and Mg: C (1.30% and 0.40%), T1 (1.79% and 0.45%), T2 (1.90% and 0.46%), T3 (2.09% and 0.47%) and T4 (2.27% and 0.50%), respectively. The Ca and Mg content of the feed was measured using inductively coupled plasma-optical emission spectrometry (Optima 8000; Perkin-Elmer, Waltham, USA). The rabbits were placed in individual breeding cages and housed in 16 hr light and 8 hr dark periods. The animals were maintained on the treatment diet for three weeks and the blood samples were collected from the marginal ear vein for biochemical parameters estimation just before allowing for mating. Blood samples were collected at 0.50 dpc and 21 dpc of the third breeding for hormone estimation. The serum was harvested by allowing the blood to coagulate for 1 hr at room temperature and centrifuging at 2,500 rpm for 15 min. The supernatant was collected and stored at – 80.00 ˚C for future use.

The bodyweight of the animals was recorded at weekly intervals and three breeding cycles were carried out by adopting a 56-day rhythm extensive reproduction system.[18] The kits, bodyweight and sex (anogenital sexing method)[19] were recorded within 24 hr of kindling and again the sex was confirmed on 21 days of age. The gender of the dead kits and randomly selected live-born kits were identified using real-time PCR (StepOne; Applied Biosystems, Waltham, USA) by the presence of *SRY* gene. At the end of the trail, the animals were deprived of food overnight and given full access to water. The oviductal samples were collected by sacrificing the animals by Sodium thiopentone anesthesia (50.00 mg kg^{-1}) for gene expression studies. The study was carried out for seven months. The study was conducted in accordance with the institutional animal ethics committee (NIANP/IAEC/1/2019) of ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India.

Gender identification. The genomic DNA from the kit tail was isolated using the TRI reagent (TRIzol) according to the manufacturer's instructions (Sigma Aldrich, St. Louis, USA). The qPCR reaction was carried out using TB Green Premix Ex Taq (Takara, Kusatsu, Japan) as per the manufacturer's instructions. The sex of the rabbit kits was identified by employing primers specific to *SRY* (Accession No. NM_001082253.1, F- 5' TACAGACCTCGTCG GAAGGT 3' and R- 5' TCTTGCCAGCTTGTCCAGTT 3' - 212bp) and *GAPDH* (Accession No. NM_001171148.1, F- 5' TGGAGAAAGCTGCTAAGTATG 3' and R- 5' CACAAAGTGG TCATTGAGGG 3' -179 bp) as house-keeping gene against the genomic DNA of the kits. The obtained PCR product was subjected to agarose gel (2.00%) electrophoresis (Bio-Rad, California, USA), (Fig. 1).

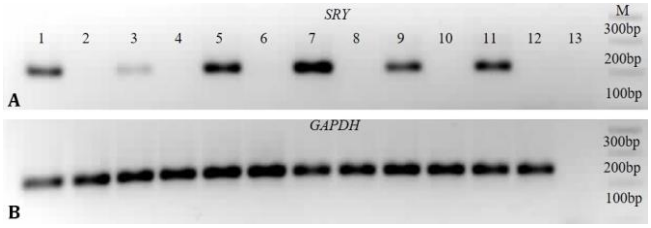

Fig. 1. Gender identification of the rabbit kits by amplification of *SRY* gene. **A)** Lanes 1, 3, 5, 7, 9, and 11 show an amplified product for *SRY,* confirming the presence of male kits, and lanes 2, 4, 6, 8, 10, and 12 having no amplified product confirms the presence of female kits. **B)** The corresponding samples show an amplified product for the housekeeping gene (*GAPDH*). Lane 13 is a non-template control.

Biochemical parameters of serum. Biochemical parameters were measured in serum collected before breeding. The glucose and cholesterol were measured using a commercially available kit (Auto span, Liquid Gold; Arkray Healthcare (P) Ltd., Ahmedabad, India). The absorbance for each of the parameters was measured using a microplate spectrophotometer (Multiskan FC; Thermo Fisher Scientific, Vantaa, Finland) at 505 nm. Serum Ca was determined using the calcium kit (Arsenazo

III Lab Care Diagnostics, Delhi, India). The Ca reacted with Arsenazo III forming a complex that shifted the absorbance which was directly proportional to the Ca concentration. The absorbance was measured at 630 nm on the microplate spectrophotometer. Serum Mg was determined by Xylidyl blue method (Proton Biologicals India Pvt. Ltd., Bengaluru, India) according to the manufacturer's instructions while the absorbance was measured on the microplate spectrophotometer at 505 nm. Serum electrolytes such as Na, K and Chloride (Cl) were measured using a commercially available kit (TRUEchemie, Bengaluru, India). The absorbance was measured on the microplate photometer at 630 nm for Na, 510 nm for Cl and 620 nm for K.

Hormone estimation. Sandwich enzyme-linked immunosorbent assay (ELISA) was carried out to estimate the serum estradiol using rabbit estradiol ELISA kit (Puregene; Genetix Biotech Asia Pvt. Ltd., New Delhi, India) and the detection levels ranged between 3.00 pg mL^{-1} to 900 pg mL^{-1} had a sensitivity of 1.54 pg mL^{-1}. The serum testosterone concentration was estimated by sandwich ELISA using rabbit testosterone ELISA Kit (Puregene) and the standard curve ranged between 0.20 ng mL^{-1} to 60.00 ng mL^{-1} had a sensitivity of 0.095 ng mL^{-1}. Solid-phase competitive ELISA (Calbiotech, Austin, USA) was used to measure the progesterone concentration at 0.50 dpc and 21 dpc. The standard curve ranged between 0 ng mL^{-1} to 60.00 ng mL^{-1} had an assay sensitivity of 0.112 ng mL^{-1}. The absorbances were measured at 450 nm using the microplate photometer.

Gene expression studies. Total RNA was isolated from oviductal samples (30.00 mg) using trizol reagent (Thermo Fisher Scientific, Waltham, USA) as per the kit protocol with slight modifications. Oviductal samples (30.00 mg) were homogenized with 1.00 mL of trizol and incubated for 10 min at room temperature followed by the addition of 200 µL chloroform. Then, it was mixed vigorously for 15 min and incubated for 3 min at room temperature followed by centrifugation at 12,000 *g* for 20 min at 4.00 ˚C. The upper aqueous layer was carefully separated and an equal amount of isopropanol was added, mixed and incubated for 10 min at room temperature. The samples were centrifuged at 12,000 *g* for 10 min and the supernatant was discarded. The pellet was washed by adding 70.00% ethanol followed by centrifuging for 5 min at 12,000 *g*. The supernatant was carefully discarded without disturbing the pellet and resuspended in 50.00 µL of nuclease-free water. The RNA yield and quality were checked by spectrophotometer (Nanodrop 1,000, Thermo Fisher Scientific). The DNase treatment was carried out using a DNA-free kit (TURBO™ DNase; Ambion, Austin, USA). An equal quantity of RNA samples was added with TURBO™ DNase buffer (0.10 vol), 1.00 µL of TURBO™ DNase and incubated for 30 min at 37.00˚C. DNase inactivation reagent was added and incubated for 5 min and mixed regularly followed by centrifuging at 1,000 *g* for 1 min. The clear supernatant was transferred to a fresh vial and the RNA concentration was measured. The cDNA was synthesized using Revert Aid cDNA synthesis kit (Thermo Fisher Scientific). The RNA sample, 10.00 µM oligo dT, and nuclease-free water were mixed and incubated for 5 min at 65.00 ˚C. The sample vials were immediately placed on ice and 5x reaction buffer, 1.00 mM dNTP, ribonuclease inhibitor and 1.00 µL of Revertaid-reverse transcriptase to make a final volume of 20.00 µL. The mixture was incubated at 42.00 ˚C for 60 min followed by 72.00 ˚C for 5 min. The samples were stored for future analysis. Primer designing was carried out using primer-BLAST (Primer3; NIH, National Library of Medicine, National Centre for Biotechnology Information, Rockville Pike Bethesda, USA) for a total of 13 functionally relevant genes (Table 1). The reaction mixture was prepared using SYBR Green Mastermix (TB Green Premix Ex Taq II, Takara, Kusatsu, Japan). The reaction was set as: Initial denaturation at 95.00 ˚C for two min, 95.00 ˚C for 5 sec (40 cycles), 61.00 ˚C for 10 sec and extension for 15 sec at 72.00 ˚C in Real-Time PCR (Applied Biosystems). Test gene expression levels were normalized to *EIF4A2* expression. Gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method[20] and the significance was tested using Student's *t*-test.

Pathway analysis of differential gene expression. The genes expression was compared between the control and T3 groups. The differentially expressed genes were subjected to gene ontology enrichment analysis using ShinyGO (version 0.741; South Dakota State University, Brookings, USA). The best matching species was set as human and the *p*-value cut-off was set to .05.

Statistical analysis. The body weight, gestational length, litters size, hormonal levels, biochemical parameters and sex ratio were analyzed by one-way analysis of variance (ANOVA) with LSD using SPSS Software (version 20.0; IBM Corp., Armonk, USA). The relative expression of the genes was correlated with the female sex ratio using the Pearson correlation coefficient. Data were expressed as mean ± SEM, and the values were considered to be significant at $p < 0.05$ and $p < 0.01$.

Results

Effect of Ca and Mg supplemented diets on the bodyweight changes of the rabbits. The mean bodyweight of the rabbits at the beginning and end of the trial was 2.59 ± 0.09 kg and 2.42 ± 0.07 kg, respectively. There were no significant changes in the mean bodyweight of the animals between different treatment groups.

Effect of Ca and Mg supplemented diets on gestation length, litter size, kit body weight and sex ratio of the kits. The gestation length and body weight of

rabbits did not vary among the treatment groups. Litter size did not differ among the treatment groups except for the experimental group T3 that the litter size in the second breeding had a significant increase (8.00 ± 0.41; $p < 0.05$). The T3 group significantly skewed the sex ratio towards females in all three breeding in an increasing trend (sex ratios 0.60, 0.67 and 0.69) and the proportion of females in the T3 group was significantly higher than that of other supplemented groups (Table 2). The experiment group T2 and T5 also skewed the sex ratio toward females in the third breeding (sex ratios 0.59 and 0.54, respectively). The mean bodyweight of the kits at birth (n = 15) for all the 3 breeding cycles was increased in a dose-dependent manner and T3 (49.52 ± 1.00 g; $p < 0.01$) and T4 (47.71 ± 0.79 g; $p < 0.05$) groups showed a significant increase (Fig. 2).

Table 1. The list of primers used for gene expression studies.

Genes	Primer sequence (5' – 3')	Product length (bp)	Accession No.
TRPV4	F: CCTATGGCCCCGTCTACTCT R: AACGAGACCGCTCCAAACTT	184	XM_008252919.2
OVGP1	F: CCCGAAGACCCCAAATCCTC R: AAGCATAGGGCACGTACTGG	271	NM_001082105.1
S100A10	F: TGAAGGACCTAGACCAGTGC R: TCTGCTTCATGTGCACTACA	114	NM_001082163.1
CCT4	F: TGACCCAGCCACAGCTACTA R: CGGTCCATCTGGGCATAGTC	237	XM_008254357.2
TCP1	F: ACCTCCGTGGTCATTATCGC R: TCTCTCCCGAGTTCATCCGT	167	NM_001163114.1
ANXA2	F: GCCTACGGCAACTTTGATGC R: CTCTGGTAGGCGAAAGCGAT	146	XM_017347978.1
ATP2A1	F: AAAGTCCCTGCAGACATCCG R: GCGATGTTGGTACCCGAGAA	173	NM_001089318.1
ESR2	R: CTTGCAGGAAGTGGACCCAT F: GGTGGGCAGTGACGATAACA	159	XM_017349392.1
IL-8	F: AAGTGGGTGCAGAAGGTTGT R: GCCCTACGACAGATCCATGC	166	NM_001082293.1
MAP2K1	F: CTACAGCGATGGCGAGATCA R: AGGATGTTGGAGGGCTTCAC	192	NM_001082629.1
TLR4	F: TGTGTGGAGGTCGTTCCCAATA R: AGGCCTTGGTATGCATCATCT	218	NM_001082732.2
TRPV6	F: GGATGAGCTGGGCCATTTCT R: CAGTGAGTGTCGCCCATCAT	198	NM_001082776.1
SLC30A7	F: GGCAAAGAAGATGTTGCCCC R: AGTTGCTCCAGATGCCGTAG	191	XM_008264812.2
EIF4A2	F: CGATGGTGTCATCGAGAGCAA R: GTGGCTGTCTTGCCAGTACC	198	XM_002716453.3

Table 2. Effect of Ca and Mg supplemented diet on reproductive performances of the rabbits and attributes of their kits at birth and weaning. Data are presented as Mean ± SE.

Groups	Parity	Weight at breeding (kg)	Gestation length (days)	Litter size (n)	Fraction of female kits	Fraction of male kits
	1	2.84 ± 0.27	30.25 ± 0.48	7.25 ± 0.48	0.43	0.54
Control	2	2.69 ± 0.24	31.25 ± 0.25	6.75 ± 0.48	0.47	0.53
	3	2.55 ± 0.16	30.75 ± 0.25	7.75 ± 0.48	0.38	0.61
	1	2.52 ± 0.21	30.75 ± 0.63	7.50 ± 0.50	0.44	0.56
T1	2	2.59 ± 0.31	30.75 ± 0.25	7.25 ± 0.25	0.52	0.48
	3	2.29 ± 0.04	30.25 ± 1.11	6.75 ± 0.48	0.59*	0.41*
	1	2.34 ± 0.20	30.00 ± 1.68	6.75 ± 0.85	0.49	0.51
T2	2	2.26 ± 0.14	31.75 ± 0.25	7.25 ± 0.25	0.55	0.45
	3	2.48 ± 0.18	30.50 ± 0.96	8.25 ± 0.85	0.43	0.57
	1	2.66 ± 0.12	31.75 ± 0.25	6.00 ± 0.41	0.60*	0.40*
T3	2	2.53 ± 0.09	31.50 ± 0.29	8.00 ± 0.41*	0.67*	0.33*
	3	2.38 ± 0.13	31.00 ± 0.41	6.00 ± 0.91	0.69*	0.31*
	1	2.60 ± 0.29	31.25 ± 0.25	7.50 ± 0.29	0.53	0.42
T4	2	2.64 ± 0.24	31.75 ± 0.25	7.75 ± 0.48	0.58	0.47
	3	2.47 ± 0.07	30.00 ± 0.82	7.25 ± 0.63	0.54*	0.46*

C: control, T1: 0.40% Ca and 0.01% Mg), T2: (0.60% Ca and 0.02% Mg), T3: (0.80% Ca and 0.03% Mg) and T4: (1.00% Ca and 0.04% Mg).
* A significant difference was determined at $p < 0.05$ when compared to control.

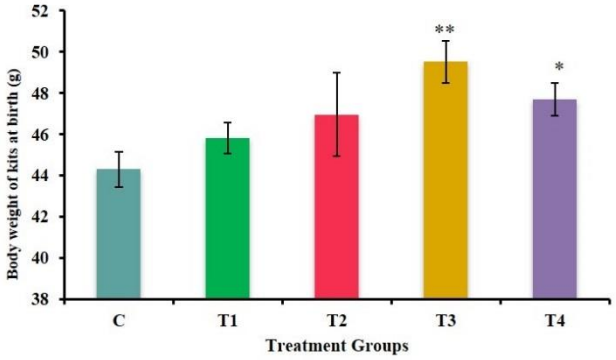

Fig. 2. Effect of maternal dietary Ca and Mg on body weight of kits. Mean body weight of kits at birth. Significant difference at * $p < 0.05$, ** $p < 0.01$, when compared to control.

The effect of Ca and Mg supplemented diets on the biochemical parameters of the rabbits. Serum Ca concentration was higher across all the treatment groups. T3 (15.26 ± 0.77 mg dL^{-1}) and T4 (15.61 ± 0.82 mg dL^{-1}) groups showed significant increase ($p < 0.05$) as compared to control (13.05 ± 0.18 mg dL^{-1}), respectively. The other biochemical parameters including K, Na, Cl, Mg, glucose and cholesterol did not differ significantly (Table 3).

The effect of Ca and Mg supplemented diets on serum estradiol, testosterone and progesterone of the rabbits. The concentration of estradiol was significantly higher in T3 (311.54 ± 13.45 pg mL^{-1}), and T4 (349.00 ± 13.10 pg mL^{-1}) groups at 0.50 dpc compared to the control (252.57 ± 18.00 pg mL^{-1}). At 21dpc, T2 (410.08 ± 6.63 pg mL^{-1}), T3 (468.75 ± 15.99 pg mL^{-1}), and T4 groups (437.22 ± 12.41 pg mL^{-1}) had significantly higher estradiol compared to the control (348.81 ± 16.78 pg mL^{-1}; $p < 0.05$), (Fig. 3A). The observed mean testosterone concentration at 0.50 dpc in the T4 group (2.72 ± 0.19 ng mL^{-1}) was significantly higher compared to the control (2.12 ± 0.15 ng mL^{-1}). At 21 dpc, T2 (3.53 ± 0.12 ng mL^{-1}) and T4 groups (3.61 ± 0.17 ng mL^{-1}) had significantly ($p < 0.05$) higher testosterone concentration compared to the control (2.93 ± 0.24 ng mL^{-1}), (Fig. 3B). Progesterone levels did not differ much among the treatment groups (Fig. 3C). T2 group had non-significantly higher progesterone concentration (1.89 ± 0.57 ng mL^{-1}) compared to the control (1.01 ± 0.08 ng mL^{-1}) at 0.50 dpc. T1 group had a non-significantly low progesterone concentration (10.60 ± 0.75 ng mL^{-1}) at 21 dpc compared to the control (11.82 ± 1.37 ng mL^{-1}).

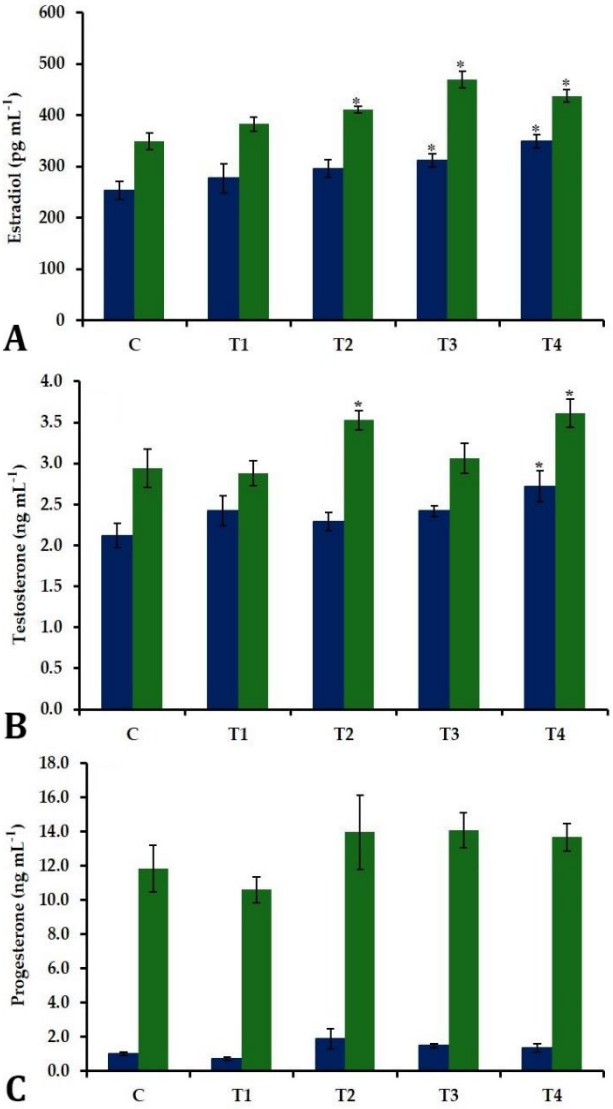

Fig 3. Effect of maternal dietary Ca and Mg on steroid hormones. Serum **A)** estradiol, **B)** testosterone and **C)** progesterone were analyzed at 0.50 dpc (blue bars) and 21 dpc (green bars) of the third breeding cycle. Significant difference at * $p < 0.05$ when compared to control.

Table 3. The baseline serum biochemical parameters of the rabbits before their mating. Data are presented as Mean ± SE.

Groups	C	T1	T2	T3	T4
Na (mmol L^{-1})	142.43 ± 2.84	140.16 ± 1.87	141.81 ± 6.01	141.40 ± 1.13	141.09 ± 8.05
Cl (mmol L^{-1})	92.77 ± 1.65	91.06 ± 2.67	93.47 ± 1.98	90.52 ± 1.36	90.31 ± 0.85
K (mmol L^{-1})	4.94 ± 0.52	4.43 ± 00.43	4.44 ± 0.07	4.58 ± 0.14	4.69 ± 0.21
Ca (mg dL^{-1})	13.05 ± 0.18	14.61 ± 0.48	14.20 ± 0.43	15.26 ± 0.77*	15.61 ± 0.82*
Mg (mg dL^{-1})	1.79 ± 0.19	1.97 ± 0.22	2.51 ± 0.15	2.13 ± 0.54	2.39 ± 0.17
Glucose (mg dL^{-1})	101.02 ± 4.44	99.25 ± 6.02	101.30 ± 5.10	93.05 ± 4.11	109.27 ± 7.37
Cholesterol (mg dL^{-1})	69.55 ± 4.55	64.57 ± 1.66	64.57 ± 5.08	63.38 ± 0.87	65.04 ± 1.00

C: Control, T1: 0.40% Ca and 0.01% Mg), T2: (0.60% Ca and 0.02% Mg), T3: (0.80% Ca and 0.03% Mg) and T4: (1.00% Ca and 0.04% Mg). Significant difference at was determined at * $p < 0.05$ when compared to control.

Differential gene expression and functional annotation. The relative expression of the selected 13 genes was compared between T3 and the control group. The normalized expression for the genes *ESR2, ATP2A1, MAP2K1, TLR4, TCP1, OVGP1, CCT4* and *ANXA2* was higher and *IL-8, TRPV4,* and *S100A10* were lower in the T3 group compared to the control (Fig. 4). Among these genes, the expression levels of *ATP2A1* (3.01-fold), *MAP2K1* (2.52-fold), *TLR4* (6.33-fold), *OVGP1* (25.84-fold), *CCT4* (26.11-fold) and *ANXA2* (23.81-fold) were up-regulated and *IL-8* (0.19-fold) was significantly down-regulated. The gene expression from all the groups was correlated with the female sex ratio. Genes such as *TLR4* ($r = 0.461$, $p = 0.041$), *OVGP1* ($r = 0.778$, $p = 0.00$), *CCT4* ($r = 0.622$, $p = 0.003$) and *ANXA2* ($r = 0.638$, $p = 0.002$) were positively correlated with the female sex ratio. The up-regulated genes of the T3 group were subject to gene enrichment analysis using ShinyGO and revealed that these genes were involved in major pathways such as toll-like receptor signaling pathway (*MAP2K1* and *TLR4,* enrichment False Discovery Rate (*FDR*) = *2.01*), *CGMP-PKG* signaling pathway (*MAP2K1* and *ATP2A1,* enrichment FDR = 1.97), Neutrophil extra-cellular trap formation (*MAP2K1* and *TLR4*, enrichment FDR = 1.92), CAMP signaling pathway (*ATP2A1* and *MAP2K1,* enrichment FDR = 1.91), PI3K-Akt signaling pathway (*MAP2K1* and *TLR4,* enrichment FDR = 1.54), ErbB signaling pathway (*MAP2K1,* enrichment FDR = 1.34). Molecular functions included carbohydrate derivative binding (*OVGP1, TLR4, CCT4, MAP2K1* and *ATP2A1* enrichment FDR = 1.95), MAP-kinase scaffold activity (*MAP2K1,* enrichment FDR = 1.56), chitinase activity (*OVGP1,* enrichment FDR = 1.60), and cadherin binding involved in cell-cell adhesion (*ANXA2,* enrichment FDR = 1.54), (Fig. 5).

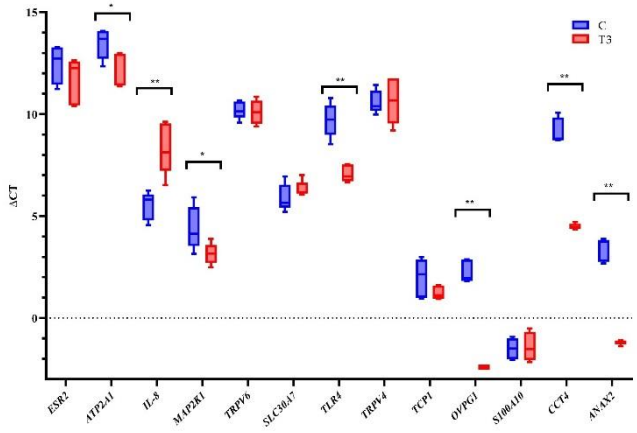

Fig. 4. Effect of maternal dietary Ca and Mg on relative expression levels (ΔCT) between control and T3 groups. The changes in CT values were normalized with the housekeeping gene (*EIF4A2*). Significant difference at * $p < 0.05$, ** $p < 0.01$, when compared to control group. The expression of genes *ATP2A1, MAP2K1, TLR4, OVGP1, CCT4* and *ANXA2* were significantly up-regulated and *IL-8* were significantly down-regulated in the T3 group compared to the control.

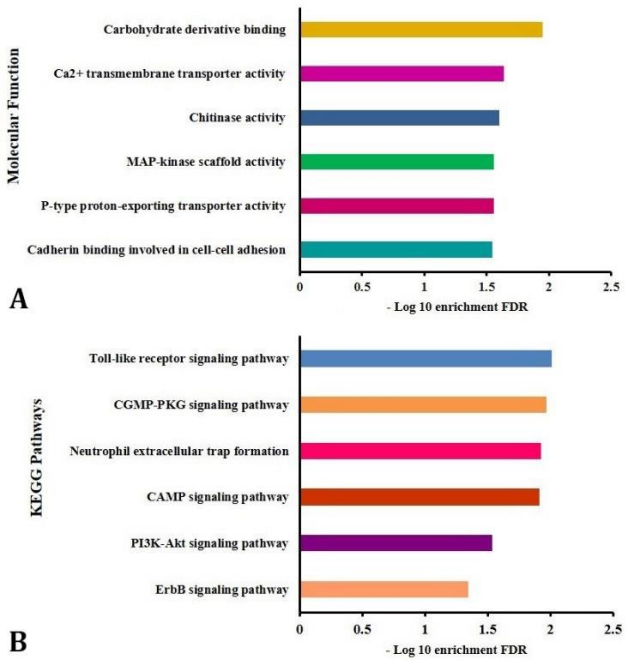

Fig. 5. Gene ontological functions and pathways enriched in the oviduct of rabbits. **A)** The functions of the genes were associated with ion transport, sperm selection and fertilization. **B)** The majority of the pathways were related to calcium signaling, fertilization, implantation, early embryonic development and immune regulation.

Discussion

Production of the desired sex of the offspring for the meat or dairy industry would be highly beneficial. Hence, it necessitates the production of more cows for milk and more bulls for meat production. In this study, the impact of different combinations of Ca and Mg intake on reproductive performances and alteration of secondary sex ratio of rabbit kits were studied in multiple extensive breeding cycles. Further trials with larger animals and complete gene sequencing of the oviduct would lead to an in-depth understanding at the molecular level. The bodyweight of the rabbits supplemented with minerals did was not significantly changed throughout the trial period.[11,21]

The sex ratio of the rabbit kits differed due to the supplementation of different levels of the combination of Ca and Mg in the maternal diets. The sex ratio of kits kindled to dams of the experimental group T3 skewed towards females in all three breeding consistently indicating that a particular concentration of Ca and Mg is critical for skewing of sex ratio. The mean litter weight at birth was increased as the Ca and Mg percentage increased in the maternal diets.[22,23]

The impact of dietary Ca and Mg supplementation on the hormonal levels was complex. Rats supplemented with Ca and vitamin D was reported to have a significantly

higher estradiol concentration.[24] In our study, Ca and Mg supplementation increased the estradiol concentration in the treatment groups. The estradiol levels were significantly higher in the group (T3) which gave female-biased litter at early and later stages of pregnancy. Similarly, mice fed with a low-fat diet to produce female-biased litter were reported to have significantly higher estradiol levels at the later stages of pregnancy.[8] In addition, increased urinary estradiol in female mouse lemurs was found to be associated with female-biased litters.[15] Estradiol administration skewed the sex ratio towards females in juvenile common snook.[25]

Supplementation of Ca and Mg increased the concentration of testosterone in the treatment groups compared to control. Similarly, in a study conducted in humans, supplementing Ca to athletes usually leads to increased testosterone levels.[26] The maternal dominance hypothesis indicates that a female dominant with higher testosterone concentration would give rise to more male offspring.[27] In contrast, elevated maternal testosterone levels are associated with the female-biased litters in Nutria.[28] There may be multiple processes acting together in the sex-selection process.

The serum biochemical profiles of rabbits were reported to be within the normal range in all the experimental groups.[29] We observed a low concentration of Na, K, Cl and higher concentration of Ca and Mg in the groups which gave birth to more female offspring (T3 and T4). The reduction in sodium chloride levels and increase in Ca and Mg levels in the animals maintained on the Ca and Mg diet in our study was consistent with the earlier studies.[11,30]

As so far, no molecular study has been conducted in this area, hence, further in-depth research is necessary to understand the effects of mineral supplementation on the female reproductive system. Additional investigation will pave the way towards understanding and application of minerals to the skewing process. The oviduct is the dynamic organ in which fertilization takes place. The oviductal secretory cells of the oviduct secrete important proteins to create a conducive environment for major reproductive events like sperm capacitation, fertilization and the initial stages of embryo development. Genes related to Ca and Mg that are important for fertilization were studied in the oviduct and correlated with the female sex ratio. When compared between the control and T3 group which produced a female-biased litter, six genes were significantly up-regulated (*ATP2A1, MAP2K1, TLR4, OVGP1, CCT4,* and *ANXA2*) and *IL-8* was down-regulated. Mineral supplementation has a beneficial effect on inflammatory markers. The *IL-8* gene expression was significantly down-regulated in all the treatment groups. Magnesium supplementation resulted in the down-regulation of *IL-8* in humans.[31] The pathway analysis of the up-regulated genes suggests their association with the fertilization processes. For instance, the toll-like receptor signaling pathway may help in immune defense in the oviduct.[32] The ErbB signaling pathway is involved in cell proliferation, cell differentiation[33] and maintenance of mature reproductive tract function.[34] Calcium regulates the PI3K-Akt signaling pathway and is involved in the embryo implantation process in rats.[35] The CAMP signaling pathway may have a key role in regulating fertilization, early embryo development and sperm function in the oviduct.[36] Neutrophil extracellular traps are formed in the female reproductive system as part of a defence system to trap the invading microbes.[37] The molecular functions of these up-regulated genes include MAP-kinase scaffold activity, carbohydrate derivative binding, cadherin binding involved in cell-cell adhesion, and chitinase activity signifies their role in the successful fertilization. Carbohydrate derivative binding activity assists in sperm-oviductal epithelial cell binding for the sperm reservoir formation.[38] Cadherin binding is also involved in cell-cell adhesion for the binding of sperm to the oviduct.[39] MAP-kinase scaffold activity is involved in cell signaling related to cell differentiation, cell proliferation and apoptosis.[40] The chitinase-like domain of OVGP1 binds to the carbohydrate moiety of zona pellucida[41] and shields the oocyte from early embryo attacks.[42]

The up-regulated genes such as *TLR4, OVGP1, CCT4,* and *ANXA2* were positively correlated with the sex ratio. The correlated genes have important functions related to sperm selection, sperm-oocyte binding, and fertilization. *ANXA2* is a calcium-regulated membrane and phospholipid-binding protein and is mainly found in ciliated cells of the oviductal epithelium.[43] It also has been involved in forming a sperm reservoir by influencing the binding of sperm to the oviduct.[44] The OVGP1 is one of the major components contributing to early reproductive events including sperm capacitation,[45] sperm-egg binding and early embryonic development.[46] The CCT4 is a chaperonin that assists in the folding of protein upon ATP hydrolysis. It is involved in the binding of sperm to the oocyte membrane.[47] The *TLR4* gene encodes for TLR4 transmembrane receptor whose activation leads to the cytokine production leading to activation of the innate immune system.[48] Sperm storage in the female reproductive tract is necessary and beneficial for the survival of a variety of species like amphibians, reptiles, mammals, birds, insects and fishes.[49] TLR4 modulates immune tolerance and protects the sperm from infection in the female reproductive tract.[50] All these molecular functions and pathways of up-regulated genes were suggestive of their role in the fertilization process which was involved in sperm selection, sperm storage, implantation, immune regulation and fertilization.

The results obtained from this study indicated that supplementation of a particular concentration of Ca and Mg led to increased production of more female offspring in

rabbits and the changes in the serum biochemical profile, steroid hormones, and gene expression were identified as parts of the underlying mechanisms altered for the production of more females. Genes such as *OVGP1, CCT4, ANXA2,* and *TLR4* were several folds up-regulated and positively correlated with the female sex ratio. These genes were involved in important functions related to sperm selection and might have a direct or indirect role in the skewing process.

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Conflict of interest

There is no conflict of interest.

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