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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

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ABSTRACT

An attempt was made with 2% calcium and 0.4% magnesium administration to assess the changes in sex ratio skewing in Wistar rat. The results indicated that there was a significant change in sex ratio skewing towards females (11.7% and sex ratio skewed was 1:1.3). There was a significant reduction in serum testosterone levels (P< 0.01) between control (0.35±0.04 ng/ml) and treatment (0.20±0.08 ng/ml) groups and a nonsignificant decrease in follicular fluid. Significant changes were observed in serum T4 level between control (52.32±2.74 nmol/L) and treatment (33.18±3.75 nmol/L) groups. Variation in follicular fluid protein profiles were observed by 2D gel electrophoresis. Differential expression of protein with high molecular weight (52 and 80 kDa) and low pH is not present in treatment group and protein with low molecular weight (10 and 11 kDa) and high pH were observed in treatment group. The expression of transcripts for BMP-15 was 11.5 folds higher and GDF-9 was 3.5 folds higher in treatment group than control. Our results suggested that possible mechanism to favour female offspring could be reduction in serum testosterone and lowering follicular fluid protein molecular weight along with high pH (haemoglobin subunit beta-1 protein (Mr) 15.9 kDa; cytochrome b-c1 complex subunit 8 (Mr) 9.8 kDa; asparagine synthetase (Mr) 64.7 kDa; metal transporter CNNM4(Mr) 87.4 kDa) and increased mRNA expression of BMP-15 and GDF-9 in oocyte.

Key words: Calcium, Magnesium, Reproduction, Sex ratio, Testosterone, Wistar rat

Till today, the proven technology for sex selection is through flow cytometry activated cell sorting, in which the selection of desired sex is reported to be >90%. However, the major constraint with this method is more damage to spermatozoa leading to reduced fertility apart from high cost of equipment. Thus, any alternate technique that is cost effective, easily adoptable and efficient method which can skew the sex ratio more than 50% would be beneficial to the farm animal's productivity. Some studies demonstrated a significant variation from the expected 1:1 birth sex ratio in many mammalian species after altering pre-conceptual maternal environment (Trivers and Willard 1973, Celik *et al*. 2003, Rosenfeld and Roberts 2004, Sheldon and West 2004, Grant and Chamely 2010, Vahidi and Sheikhha 2007). We measured the changes in sex ratio skewing by estimation of mineral and steroid hormones level in rat model with calcium and magnesium administration.

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MATERIALS AND METHODS

Animal breeding and diet conditions: All procedures involving animals were carried out under license and in accordance with the Experimental Livestock Unit, Lab animal section CPCSEA, approved by the local ethical review committee of the ICAR-NIANP, Bengaluru. Sixty female (60) and male (12) Wistar *(Rattus norvegicus)* rats (180–220 g; 14–16 weeks of age) were procured commercially and housed as a group for 10 days for acclimatization. In both groups, rats were fed *ad lib.* on commercial rat chows (control diet consists of moisture 7%, crude protein 21.4%, crude fat 4.8%, crude fibre 3.15%, calcium 1.1%, phosphorus 0.50%, total ash 5.5%, carbohydrates 65% and energy 3,140 kcal/kg) and had free access to portable water. Rats were housed under controlled temperature (20–30ºC) and humidity. The rats were randomly assigned to control (group 1) and treatment (group 2) groups. The treatment calcium and magnesium were supplemented through water @ 2 and 0.4%, respectively in the treatment group. The rats were supplemented with additional Ca and Mg for 21 days comprising of 15 days precede and 7 days of mating period. At the end of 15 days of treatment, the female rats were mated (female 4: male 1)

Table 1. Gene specific primer details

Transcripts	Primer sequence	Primer	Primer length	Product size	Accession number
GAPDH	ACTTTGGCATCGTGGAAGGG	F	20	128bp	NM_017008.4
	TGCAGGGATGATGTTCTGGG	R	20		
GDF-9	ATAGGCGAAGTGAGACCCCT	F	20	193bp	NM_021672.1
	AGGGGTCCTGTCATCTGGTTG	R	21		
BMP-15	AGGCTTCTCAGAGGTCCTTGG	F	21	149bp	NM_021670.1
	CAAGGTTCCACATGGCAGGA	R	20		

with male rats and upon pregnancy females. The pups were sexed by means of ano-genital distance and the male and female ratio was calculated.

Blood collection and serum preparation: Blood and follicular fluid were collected on the day when male rats were allowed for mating (i.e on 15 day) of mineral administration after sacrificing rats using ether anaesthesia. Non-heparinized whole blood sample were collected by cardiac puncture, and serum separated, aliquoted and stored at –20ºC. Follicular fluid was collected and stored at –20ºC. Estradiol, testosterone, triiodo thyronine (T3) and thyroxine (T4) and minerals were estimated in serum and follicular fluid using radioimmunoassay and inductively coupled plasma mass spectrophotometry. Following aspiration of follicular fluids, the oocytes were recovered as per Eppig *et al*. (1985) and stored at –80ºC till further analysis.

Analysis of hormone and minerals: Steroid hormones (estradiol, testosterone) and triiodothyronine (T3) and thyroxine (T4) levels were estimated using radioimmunoassay (RIA) with commercially available diagnostic kits following manufacturer's instructions.

Mineral: Macro and micro mineral level (Ca, Mg, B, Cu, Fe) were estimated in serum and follicular fluid by inductively coupled plasma mass spectrophotometry.

2-D gel electrophoresis of follicular fluid proteins: The concentration of proteins in follicular fluid was estimated using spectrophotometer (Thermo) and 50 µg of proteins was mixed in sample buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% biolyte ampholyte, 2 M thiourea) and final volume of 125 µl per well in IEF plate was loaded. The entire procedure involved in running the 2-D gel electrophoresis were followed as per the earlier described methods (Gromova *et al*. 2006, Olivier *et al*. 2010, Arangasamy *et al*. 2015). Precast immobilized pH gradients strips (IPG Strips 7cm, pH 3–10) and 8–16% precast gels were used in this study. The molecular weight, raw volume and quantity of separated proteins were analyzed using Dimension 2D gel analysis software to study the differential expression of protein between the two groups.

Protein sequencing and analysis: The differentially expressed proteins between treatment (spot numbers1–6) and control group (spot number 7 and 8) were subjected to MALDI-TOF analysis after trypsin digestion. The MALDI-TOF peptide mass results were analyzed through Mascot search engine (www.matrixscience.com) as per Arangasamy *et al*. (2015).

Total RNA extraction from oocyte and primer designing: Oocyte from each of treatment and control group (40–50 oocyte) was pooled separately and washed with phosphate buffered saline (PBS, pH - 7.4) by centrifuging at 1,000 g for 10 min. Oocyte pellets were suspended with PBS and centrifuged again at the same conditions (3 washing steps). Then, total RNA was isolated from the oocyte pellet, using commercially available RNA kit and eluted in 30 µl RNase free-water as per the standard procedure given by the manufacturer. The RNA concentration was measured using a NanoDrop spectrophotometer. Sample absorbance ratio of 260/280 wave-length was determined to ensure the purity of RNA (target of close to 2.00). The RNA samples were stored at –80°C till cDNA preparation. The gene specific primers for qRT-PCR analysis were designed from *Rattus norvegicus* as per the details given in Table 1.

Polymerase chain reaction of selected genes of interest: The mRNA was reverse-transcribed to cDNA. The cDNA samples were prepared using the reverse transcriptase. The designed primers were optimized prior to quantification experiments using polymerase chain reaction.

Determination of mRNA expression using real-time PCR: The SYBR green approach was used to determine relative mRNA expression. SYBR Green master mix (Cat No. K0221) was used to prepare the reaction mix. Two technical replicates (25 µL each) were used for each sample, with 6 ng/reaction RNA equivalent cDNA present in each, and a final primer concentration of 0.25 µM. StepOne Plus instrument was used for real time PCR. The PCR cyclic conditions were 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and then annealing at 60°C for 1 min. The baseline was automatically adjusted to obtain threshold cycles of each sample. Threshold cycles were normalized to an endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences, expected fragment size of amplified products and genbank accession numbers are given in Table 2. For these genes, the expected sizes of the products were confirmed by gel electrophoresis on a 1.2% agarose gel and stained with ethidium bromide for visualization to ensure a single amplicon for a set of primers.

Interpretation of qRT-PCR and statistical analysis: Data were quantified with relative quantification method as described by Pfaffl (2002) using the formula $2^{-\Delta\Delta Ct}$. The amount of target transcripts relative to the calibrator was calculated by subtracting the Ct value of sample reference

gene from the Ct value of the sample target gene.

Statistical analysis: Statistical analysis of the data was done as per Snedecor and Cochran (1989). The student T test was used for analyzing the various data in the present study to check the significant level among the mean values of various parameters.

RESULTS AND DISCUSSION

In the present study, skewing of sex ratio was achieved significantly by supplementing with additional Ca and Mg (Table 2). Sex ratio was skewed towards females by 11.7% by this method (116 vs 89). A net skewing of 1:1.3 was achieved between the 2 groups. Only serum P levels showed a significant (P< 0.01) change (64.01±5.99 Vs 52.14±6.06 ppm) between treatment and control groups. Serum testosterone level was significantly (P< 0.01) lowered in treatment (0.20±0.08 ng/ml) compared to control (0.35±0.04 ng/ml) groups, but not the estradiol levels. Similarly, serum T4 but not T3 was significantly decreased (52.32±2.74 Vs 33.18±3.75 nmol/l) in treated rats. The rat follicular fluid protein pattern is presented in Fig.1; 226 protein spots were observed in treatment group and 168 protein spots were observed in control groups. Scatter plot shows differential expression of proteins between the control and the treatment groups. Supplementation of ca^{++} and mg^{++} regulates (up-regulation /down-regulation) proteins expressions that favor skewing of sex ratio. The differentially expressed proteins in treatment groups (spot number 1 and 2:10 and 11 kDa) identified as hemoglobin subunit beta-1 protein (molecular weight 15.9 kDa) and cytochrome b-c1 complex subunit 8 protein (molecular weight 9.8 kDa). Similarly, in control group the differentially expressed proteins (spot number 7 and 8:52 and 80 kDa) identified as asparagine synthetase protein (molecular weight 64.7 kDa) and metal transporter CNNM4 (molecular weight 87.4 kDa) (Fig. 1), respectively. Oocytes GDF-9, BMP-15 transcripts expression level were quantified with real time PCR and their amplified product size was confirmed with 1.2% agarose gel (Fig. 2). The mRNA expression of GDF-9 and BMP-15 in oocyte showed differential changes among treatment and control rats. The variation of BMP-15 mRNA abundance was higher (11.5 folds) in treatment group than control and the variation of GDF-9 mRNA abundance was also higher (3.5 folds) in treatment group than control.

In the present study, an attempt was made to study whether mineral (Ca, Mg) imbalance in the diet of the female before fertilization affects the sex ratio of the progeny. Our results suggested that supplementation of additional Ca, Mg can significantly (P< 0.05) alter the sex ratio in rats. However, the low level of skewing of sex ratio reported in this study compared to earlier reports (Vahidi and Sheikhha 2007, Chandraju *et al*. 2011) might be due to the levels of mineral used and/or the route of administration. The works (Khalifa *et al.* 2009, Machado *et al.* 2012)

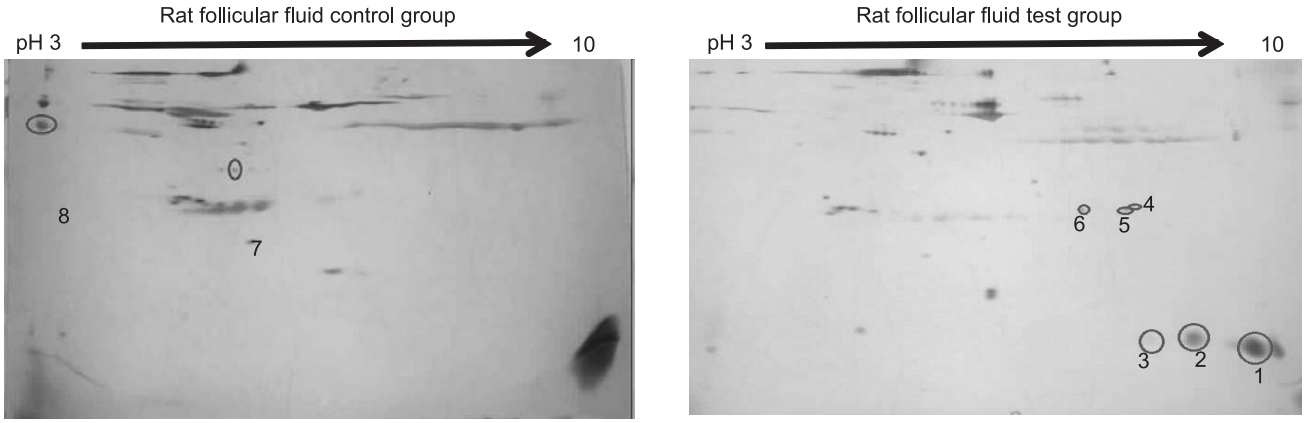

Fig.1. 2-D gel electrophoresis of rat follicular fluid proteins. Spot number 1 (haemoglobin subunit beta-1protein (Mr) 15.9 kDa), spot number 2 (cytochrome b-c1 complex subunit 8(Mr) 9.8 kDa) in treatment group; spot number 7 (asparagine synthetase (Mr) 64.7 kDa), spot number 8 (metal transporter CNNM4(Mr) 87·kDa) in control group. Supplementation of Ca^{++} and Mg^{++} regulates (up-regulation /down-regulation) proteins expressions that favor skewing of sex ratio.

Table 2. Sex ratio changes due to calcium and magnesium supplementation

Number of rats allowed for breeding	Total pups	Female pups	Male pups	Sex ratio skewed (Female:Male)
Treatment (30)	188	116[b]	72	1.3:1
Control (30)	174	89[a]	85	1:0.85

[ab]Mean bearing different superscript differ significantly in a column (P< 0.05).

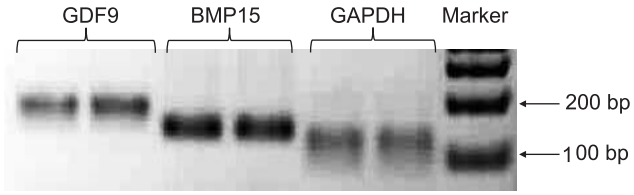

Fig. 2. RNA isolated from rat oocyte and tested for expression of target genes by PCR amplification. Expected sizes of the products were confirmed by gel electrophoresis on a 1.2% agarose gel and stained with ethidium bromide.

conducted in cattle also indicated more percent of female young one production by changing the ionic pH of semen diluents. The observed changes in the levels of steroid hormones and macro and micro mineral further support the sex allocation hypothesis (Trivers and Willard 1973). Further, serum testosterone levels significantly decreased in the rats supplemented with Ca, Mg, suggesting the probable inverse relationship between androgen and sex of offspring. Further skewing decreased the circulating levels of T4 but not T3, which is in accordance with Sosic-Jurjevic *et al*. (2006). However, the mechanisms underlying these effects are still unclear.

The expression of haemoglobin subunit beta-1 protein (Mr) 15.9 kDa) and cytochrome b-c1 complex subunit 8 (Mr) 9.8 kDa in treatment group and high molecular weight protein asparagine synthetase (Mr) 64.7 kDa and metal transporter CNNM4 (Mr) 87.kDa in control group, indicated the influence of calcium and magnesium on follicular fluid protein pattern changes towards basic side. The observed differential expression of proteins in control and treatment rat follicular fluids by Scatter plot clearly indicated that supplementation of Ca^{++} and Mg^{++} regulated protein expression to favor skewing of sex ratio. However, no supporting literature is available in this regard to compare and confirm these results. This study could be the first report on the influence of calcium and magnesium on follicular fluid protein pattern while skewing the sex ratio in rats. The relative increase in transcripts expression of GDF-9 (3.5 folds) and BMP-15 (11.5 folds) in the present study indicated that the exogenous administration of calcium and magnesium might alter oocyte genes expression while skewing the sex ratio. Similarly, over expression of BMP-15 and GDF-9 in the treatment group may be due to the probable influence of calcium and magnesium ions on oocyte maturation and/or polarity cycle of the oocyte membrane to attract particular proportion of sperm cells to bind and fertilize the oocyte. This probably could be due to the effect of Ca and Mg concentration on the metabolism and intracellular pH of fallopian tubes and changes in the ratio of ions in the ovaries and eggs. This may lead to potential changes in membrane components and structure of the sperm binding sites on the membrane of the ovum, thus facilitating binding of certain type of X or Y sperm to the ovum. Changes in the reproductive tract conditions might have differentially affected embryonic development of one sex over the other (Farhadi *et al*. 2014). Since the shift in pH of the follicular fluid from acidic to basic side during treatment might have selectively attracted negatively charged X chromosome bearing spermatozoa (Grant and Chamely 2010) thus skewing the sex ratio towards females. Further studies are required to confirm the exact roles of these proteins on oocyte growth and recognition of spermatozoa and its binding receptors for skewing sex ratio need to be explored towards more logical conclusions. Overall, it can be concluded that administration of calcium and magnesium for purposes of skewing sex ratio have effects on skewing sex ratio in rat, with a significant reduction in circulating levels of testosterone, changes in pH of follicular fluid towards basic side and enhance the expression of certain genes in oocyte.

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