

Study of the impact of organic minerals on spermatozoal gene expression in Osmanabadi bucks

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ABSTRACT

Sperm transcripts aside from providing genetic material have an eminent role in post-fertilization events and embryonic development. The current study was carried out to study the gene expression in Osmanabadi buck (*Capra hircus*) supplemented with organic minerals. Organic minerals alone or in combination with different concentrations were fed to the treatment groups (T₂- Zn 20 mg, T₃- Zn 40 mg, T₄- Zn 60 mg, T₅- Cu 12.5 mg, T₆- Cu 25 mg, T₇- Cu 37.5 mg, T₈- Zn 20 mg + Cu 12.5 mg, T₉- Zn 40 mg + Cu 25 mg, T₁₀- Zn 60 mg + Cu 37.5 mg) along with the concentration mixture and roughages; whereas control (T₁) group was fed concentrate mixture and roughages. The sperm samples were processed for gene expression studies of Ras Homolog Family Member A (RHOA), Mesoderm Specific Transcript (MEST), Nucleoside diphosphate-linked moiety X motif 6 (NUDT6), Protamine 1 (PRM1), Protamine 2 (PRM2) and Heat shock protein 90 alpha family class A member 1 (HSP90AA) using qRT-PCR and the protein-protein interaction analysis was carried out through String database version 11.0. Supplementation of trace minerals has enhanced the gene expression at a significant level (P<0.05) in the supplemented groups. The expression of MEST, RHOA, and PRM1 was higher in Cu 25-supplemented groups; whereas, NUDT6 in Zn 20, PRM2 and HSP90AA in Zn 40 showed higher expressions. Osmanabadi bucks supplemented with Cu 25 mg have shown promising results in the present and earlier studies so it can be concluded that the field-level application of Cu 25 mg would lead to promising results.

Keywords: Bucks, Copper, Fertility, Gene expression, Organic minerals, Spermatozoa, Transcript, Zinc

In the past, supplementation of organic minerals has had tremendous application in the field of livestock production. Nutrient deficiencies in soil, underfeeding, and degraded grazing lands lead to impairment in puberty and semen quality. The composition of trace minerals in the fodders depends on the soil nutrition on which it has grown, rainfall, cropping pattern, and feeding system (Mayasula *et al.* 2021). Trace minerals play a major role in many biological reactions as catalyst. The most common trace minerals used are iron, copper, zinc, manganese, chromium, and selenium. Zinc has influence on sperm membrane stability, sperm motility, maintenance of chromatin structure, and reactive oxygen species (ROS) production and regulation of apoptosis (Sekar *et al.* 2021). Copper is required for the antioxidant properties of metalloenzymes and metalloproteins which protect sperm from premature hyperactivation and capacitation (Sekar *et al.* 2021). Organic trace minerals are preferred over inorganic trace minerals due to their bioavailability

and non-toxic nature. Supplementation of organic minerals alone and combination (Zn, Cu, Cu + Zn) to bucks has improved the overall reproduction efficiency by enhancing seminal characteristics, antioxidant activity (Narasimhaiah *et al.* 2018), biochemical profile of seminal plasma (Mayasula *et al.* 2020), spermatozoal gene expression (Krishnaiah *et al.* 2019), cryotolerance and puberty (Arangasamy *et al.* 2018). Reproduction efficiency is influenced by the expression pattern of transcriptome along with the sperm morphokinetic parameters. The sperm contains a population of RNAs that contribute to various functional roles in spermatogenesis, fertility, and embryonic development (Sahoo *et al.* 2021). The earlier studies documented the higher expression of antioxidant defense enzyme genes (Arangasamy *et al.* 2018), stress-responsive genes, and fertility-related genes (Mayasula *et al.* 2021) in organic minerals-supplemented groups. The objective of this study is to investigate the influence of organic mineral supplementation on the expression pattern of RHOA, MEST, NUDT6, PRM1, PRM2, and HSP90AA.

MATERIALS AND METHODS

Management of animals and sample collection: This study was the continuation of DBT project which was carried out in the Experimental livestock unit with prior

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approval of the Institutional Animal Ethics Committee (IAEC) from December 2015 to August 2016 (Arangasamy *et al.* 2018). The five-month-old healthy male Osmanabadi breed bucks ($n=40$) with uniform body weights and size were randomly categorized into ten groups ($n=4$), each group comprising four bucks. The concentration mixture (maize 44%, wheat bran 20%, groundnut cake 17%, soya bean meal, 2% mineral mixture, and 1% salt) and roughages (50:50) were fed to the control group (T_1). Organic minerals alone or in combination with different concentrations were fed to the treatment groups (T_2 - Zn 20 mg, T_3 - Zn 40 mg, T_4 -Zn 60 mg, T_5 - Cu 12.5 mg, T_6 - Cu 25 mg, T_7 - Cu 37.5 mg, T_8 - Zn 20 mg + Cu 12.5 mg, T_9 - Zn 40 mg + Cu 25 mg, T_{10} - Zn 60 mg + Cu 37.5 mg) along with the concentration mixture and roughages per kg dry matter (DM) based on the NRC guidelines (Ranjhan 1998) for eight months. Semen samples were collected from the control and treatment groups ($40 \times 5 = 200$) using an electro-ejaculator (Arangasamy *et al.* 2018) with three days of abstinence in each collection. To the samples, protease inhibitor was added and centrifuged at 3000 rpm for 10 min at 4°C. The pellet containing sperm cells was resuspended in phosphate buffer saline (PBS pH 7.2). The snap-frozen cells were further stored at -80°C and five ejaculates from each buck were pooled and represented as one animal.

RNA isolation and DNase treatment: The total RNA was isolated using both the manual TRIzol method and membrane-based PureLink RNA Mini Kit (Ambion, USA) method as depicted by Parthipan *et al.* (2015). The snap-frozen sperm cells were thawed and resuspended in PBS at room temperature and 100 million sperm cells were used for RNA isolation. Further the quality and quantity of RNA were assessed by a spectrophotometer (Nanodrop 1000, Thermo Fisher, USA) at 260/230 ratio. DNase treatment was carried out as per the instructions given in the TURBO DNA-free Kit (Invitrogen, USA) to remove the DNA contaminant from RNA samples. The samples were further stored at -20°C until use.

cDNA synthesis and gene expression quantification: The cDNA from the total RNA samples was synthesized using

the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and the cDNA samples were stored at -80°C. Gene expression quantification was carried out using SYBR Green Mastermix (TB Green Premix Ex Taq II, Takara Bio, Japan) in real-time PCR (Step One Plus, Applied Biosystems, USA). The reaction mixture was composed of 10 µl TB Green Premix Ex Taq II, 0.8 µl of each forward and reverse primer, 0.4 µl Dye, 2 µl cDNA samples (15 ng/µl), and 6µl nuclease-free water. Primers used in the study were designed (Table 1) using primer-BLAST (Primer3; NIH, National Library of Medicine, National Centre for Biotechnology Information, Rockville Pike Bethesda, USA). The samples for the test and the endogenous control genes were prepared in triplicates with non-template control. The cycle conditions were set as: Initial denaturation at 95°C for 2 min; 40 cycles of 95°C for 5 s; 63°C for 10 s and extension for 20 sec at 72°C with default melt curve settings. The test gene expression levels were normalized to RPL23 expression and the Cq data were analyzed using StepOne software (v2.2.2).

Statistical and bioinformatics analysis: The relative gene expression was calculated as per the method adopted by Livak and Schmittgen (2001). The values were considered significantly different at $P<0.05$. The functions of gene products were studied using protein-protein interaction analysis using the open-access database-based online tool STRING (<http://string-db.org>) version 11.0 and *Homo sapiens* was assumed as the source organism. The interactions were based on co-expression, databases, text-mining, and experimental data. Proteins with a confidence score above 0.900 were selected for analysis.

RESULTS AND DISCUSSION

The study explored the gene expression pattern of Osmanabadi bucks supplemented with organic minerals (Zn, Cu, and Zn+Cu). Differential expression of RHOA, MEST, NUDT6, PRM1, PRM2, and HSP90AA genes and the protein-protein interaction of the gene products in Osmanabadi bucks which were fed with organic minerals (Zn, Cu, and Zn+Cu) were reported. The relative mRNA

Table 1. Primers used in the gene expression studies

Accession no.	Gene	Sequence	Length
XM_001286443.1	<i>RHOA</i>	FP 5'GTCGTCGCCTGAGTGCCTTT3' RP5'GTCTTGCCACAGGCTCCATC3'	150
XM_005679479.3	<i>MEST</i>	FP5'CCCCTGCTCTTCACTCATGGA3' RP5'CACACCCACAGAGTCTTGGA3'	86
XM_013970536.2	<i>NUDT6</i>	FP5'GTGGGAGTTGCAGGAGCTGT3' RP5'TGCCGAATACTCAGGAGGGA3'	200
XM_018072569.1	<i>PRM2</i>	FP5'CTCCAGCACGATGGTCCGAT3' RP5'CGAGTGTGGCTCCTGTGTCT3'	186
HM773246.1	<i>PRM1</i>	FP5'AAGATGTGCGACGAAGGAG3' RP5'GCTACTGTGCGGTTACTGTCT3'	106
XM_018066239.1	<i>HSP90AA1</i>	FP5'GCCAGTTTGGTGTGCGGCTTT3' RP5'CCCATCGGTTCTCCTGTGTCA3'	148
XM_0056409.3	<i>RPL23</i>	FP5'CAGCGGTGGTAATTCGACAAC3' RP5'GGCGGAACCTTTCATCTCG3'	116

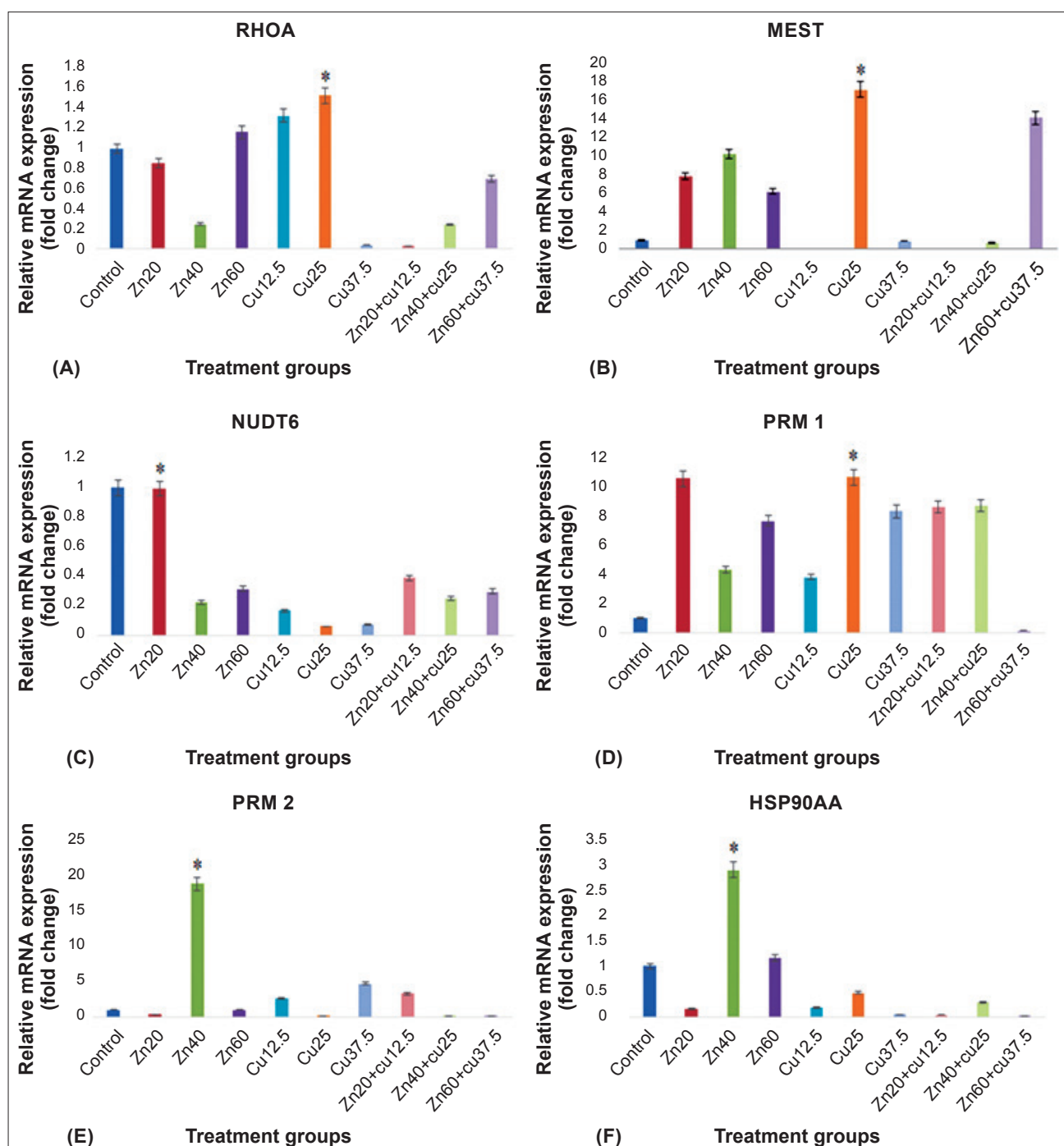


Fig. 1. The relative expression transcripts; A. *RHOA*; B. *MEST*; C. *NUDT6*; D. *PRM1*; E. *PRM2*; F. *HSP90A* in control and organic mineral supplemented groups normalized with the housekeeping gene (*RPL23*). The fold change in control group was considered as one. *Indicates significant difference, $P < 0.05$.

expression level of *RHOA* in trace mineral-supplemented groups is shown in Fig. 1A. The expression level was significantly higher ($P < 0.05$) in all the groups except the Zn 20 + Cu 12.5 supplemented group where the gene expression was significantly ($P > 0.05$) lower (0.03). *RHOA*, a RHO protein, has diverse role in capacitation reaction by cytoskeleton remodelling in bovine via CDC42 activity and GNA13-mediated *RHOA*-ROCK2-LIMK2-cofilin signalling pathway (Reyes-Miguel *et al.* 2020). In the present study, we observed higher expression of *RHOA* in

groups fed with Cu 25 mg, Cu 12.5 mg, and Zn 60 mg (fold change - 1.53, 1.33, and 1.16). Earlier work done in same laboratory has also reported a higher number of sperm cells bound to zona in Cu-supplemented groups (Cu 12.5 mg - 26.83 ± 1.23 , Cu 25 mg - 22.06 ± 1.98 , and Cu 37.5 mg - 22.85 ± 1.20) (Hemalatha *et al.* 2018). From the above observations, it can be assumed that the higher expression of *RHOA* in Cu supplemented group might be associated with the sperm-egg fusion by remodelling the cytoskeleton for a successful acrosome reaction.

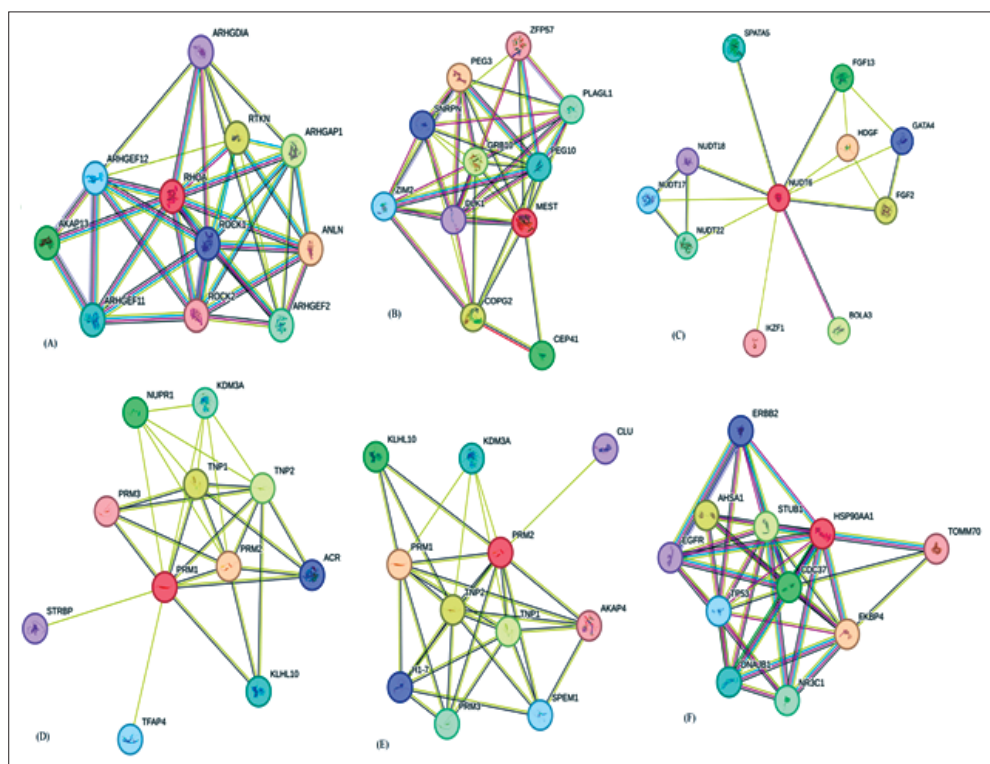


Fig. 2. Protein-protein interaction analysis of the products of; A. *RHOA*; B. *MEST*; C. *NUDT6*; D. *PRM1*; E. *PRM2*; F. *HSP90A*.

MEST maternally imprinted gene methylated and repressed in female germ cells but expressed (remains unmethylated) in male germ cells. Hypermethylation of *MEST* has led to spermatogenesis failure, defective post-fertilization development, growth retardation, and recurrent pregnancy loss (Rotondo *et al.* 2021, Khambata *et al.* 2021). The Cu 25-supplemented group has shown a higher level ($P < 0.05$) of gene expression (fold change - 17.36) of *MEST* followed by other mineral-supplemented groups Figure 1B. Whereas, the gene expression was ($P > 0.05$) lower in Cu 12.5 (0.04) supplemented group. We observed significant expression of *MEST* in all the treatment groups with the highest expression in the Cu 25 mg supplemented group (fold change - 17.36). However, the expression was significantly lower ($P > 0.05$) in the Cu 12.5 mg supplemented group. In earlier studies, the animals supplemented with organic minerals have shown promising evidence in all the sperm morpho-kinetic parameters which may be associated with the *MEST* expression. The *NUDT6* expression levels were significantly ($P < 0.05$) higher (Fig. 1C) in all the groups but lower than the control group (fold change - 1) though the actual functional role has not yet been explored. Decapping (removal of 5' cap structure) was an essential and highly regulated process for mRNA degradation to control gene expression. DCP2 and *NUDT16* have differential roles in mammalian mRNA decay pathways (Borbolis F and Syntichaki P 2022). The abundant expression of *NUDT18* may be associated with antioxidant stress during the freeze-thawing process in buck spermatozoa (Zhu *et al.* 2020). We speculate that the higher expression of *NUDT6* may be essential as the

members of Nudix hydrolases were associated with the prevention of mutations and regulation of gene expression by mRNA degradation.

Protamines (PRM1 and PRM2) were crucial for the protection of the paternal genome from internal and external impressions by densely packing the sperm chromatin in the minor groove of DNA via intermolecular and intramolecular disulphide bonds. The abnormal protamine levels may impact late spermiogenesis, sperm penetration capacity, and embryo development in which DNA integrity is preminent. The downregulation of PRM2 transcripts in male partners is associated with idiopathic recurrent miscarriage (Ren *et al.* 2021). PRM1 and PRM2 were significantly higher ($P < 0.05$) in all the groups. All the organic mineral-supplemented groups have shown (Fig. 1d) a significant level of PRM1 gene expression. However, the highest level of gene expression was observed in the Cu 25 (10.63) supplemented group. The expression level of PRM2 was higher ($P < 0.05$) in all the groups and the highest level of expression was observed in the Zn 40 (18.85) supplemented group (Fig. 1E).

Relative expression of HSP90AA is shown in Fig. 1F where the expression level was significantly ($P < 0.05$) higher except for Zn 20 + Cu 12.5 (0.04) and Zn 60 + Cu 37.5 (0.01) which was significantly ($P > 0.05$) lower. HSP90, 90-kDa heat shock protein (HSP90AA1) environmentally responsive and abundant chaperone plays a major role in spermatogenesis and sperm function. The expression of HSP90 is upregulated to compensate oxidative stress in sperm higher level of HSP90 is reported in patients with recurrent idiopathic pregnancy loss (Kumar *et al.*

2013). HSP90 involve in PRL-induced apoptosis during spermatogenesis, and regulate sperm capacitation in human via Erk1/2 and p38 MAPK signaling pathways (Sun *et al.* 2021). Narasimhaiah *et al.* (2018) reported supplementation of organic minerals to Osmanabadi bucks enhanced the sperm motility, plasma membrane, and acrosome integrities which supports our observations. HSP90 may regulate spermatogenesis and motility by neutralizing the reactive oxygen species.

The protein-protein interaction studies have shown that RHOA promotes reorganization of the actin cytoskeleton (Fig. 2A), MEST maintains maternal and paternal gene imprinting (Fig. 2B), NUDT6 regulates cell proliferation (Fig. 2C), PRM1 stabilizes sperm structure during spermatogenesis (Fig. 2D), PRM2 packaging of genetic material (Fig. 2E), and HSP90AA regulate signal transduction and cell cycle (Fig. 2F). Arangasamy *et al.* (2018) reported that organic Cu supplementation had a significant effect on sperm concentration, motility, and membrane and acrosome integrity, progressive and mass motility. Mayasula *et al.* (2020) observed changes in seminal plasma profile and reduced stress had improved the quality of sperm in the Zn 40 mg supplemented group. Sekar *et al.* (2021) reported a higher number of differentially expressed seminal plasma proteins in Cu-supplemented groups. From these observations, we strongly assume that the studied genes were vital for spermatogenesis, post-fertilization success, and embryo development.

In brief, the genes Ras Homolog Family Member A, Mesoderm Specific Transcript, and Protamine 1 were significantly expressed at higher levels in Cu 25 mg, Nucleoside diphosphate-linked moiety $\times$ motif 6 in Zn 20 mg, Protamine 2 and Heat shock protein 90 alpha family class A member 1 in Zn 40 mg. It can be concluded that among the organic minerals supplemented groups in Osmanabadi bucks Cu 25 mg had enhanced quantitative and qualitative sperm characteristics, antioxidant enzyme profile and gene, sperm-egg fusion, seminal plasma protein profile, and sperm motility. Field level application of Cu 25 mg to animals is strongly recommended.

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