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Semen quality, lipid peroxidation and expression of mitochondrial gene in ejaculated sperm of Karan Fries (Tharparkar × Holstein Friesian) bulls supplemented with astaxanthin

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ABSTRACT

This study was conducted to evaluate the effect of astaxanthin (potent herbal antioxidant) supplementation on sperm quality, lipid peroxidation and expression of mitochondrial genes in semen of Karan Fries (Tharparkar × Holstein Friesian) bulls during summer under tropical climatic conditions. Adult healthy bulls (10) were selected and divided equally into 2 groups i.e. control and treatment (supplemented astaxanthin @ 0.25 mg/kg body weight/day/animal). Ejaculates were collected at weekly interval in early-morning from bulls using artificial-vagina from April to August. Just after collection, semen samples were placed in a water bath (37°C) for semen analysis. Astaxanthin supplementation improved semen quality parameters (volume, motility, concentration, and acrosome-integrity) over non-supplemented bulls. The major abnormalities were lower in supplemented bulls. Semen malondialdehyde concentration was also lower in treatment than control group. The higher concentration of total antioxidant capacity was observed during July and August in supplemented bulls. Relative expression (mRNA) of succinate dehydrogenase, citrate synthase and mitochondrial transcription factor-A was upregulated in spermatozoa of supplemented bulls than control bulls. Supplementation of astaxanthin to crossbred bulls during summer improved the semen quality by improving the antioxidant activity and modulating the mitocondrial gene expression during the summer season in the tropical climate. Therefore, astaxanthin supplementation could be suggested for improving the semen quality of crossbred bulls during summer season.

Key words: Astaxanthin, Crossbred bulls, Mitochondrial gene, Semen quality, Summer

Astaxanthin (AX) is a xanthophyll carotenoid (colour pigment) having the powerful antioxidant ability, neutralizes free radicals or other oxidants by either accepting or donating electrons without being destroyed or becoming a pro-oxidant in the process. Several studies have revealed the beneficial health effect of AX as photoprotectants, eye health, anti-inflammatory, immunity enhancement and other important application in nutraceuticals, cosmetics, food and feed industries (Guerin *et al*. 2003). AX is more effective than beta-carotene and lutein in preventing photo-oxidation of lipids (O'Connor and O'Brien 1998). It enhances antibody production and restored to decrease humoral immune responses in old mice (Jyonouchi *et al*. 1994). AX enhances the mitochondrial activity (Wolf *et al*. 2010). Sperm motility and pregnancy rate were increased in AX supplemented group as compared to the placebo group in human (Comhaire *et al*. 2005). Tripathi and Jena (2008) also reported the improvement of testes weight, sperm concentration and sperm morphology in mice when supplemented AX with cyclophosphamide as compared to only cyclophosphamide-treated mice (induced germ cell toxicity in mice). Tripathi and Jena (2010) also reported revealed that AX can ameliorate oxidative stress, DNA damage and cell death in cyclophosphamide treated rat, and it decreased the expression of p53, p38 and increased the level of Nrf2, phase-II enzymes (NQO-1, HO-1), thus proposed a mechanism of chemoprotection through NRF2-ARE pathway.

Heat stress is one of the major threats to animal production system under tropical climatic conditions (Shelton 2000). Hence, strategies are essential to combat the adverse effect of climate change on animal production and reproduction. Hence, adverse climatic conditions in tropical areas lead to oxidative stress, mitochondrial dysfunction which resulted in poor sperm quality. The supplementation of AX @ 2 and 4 µM was found to increase sperm vitality and plasma membrane integrity during the storage period (72 h) (rams' liquid semen) at 4°C, besides reduction in lipid peroxidation and reactive oxygen species (ROS) levels (Fang *et al*. 2015). Higher sperm motility and

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fertilization rate were also observed in AX supplemented group in goldfish (*Carassius auratus*) (Tizkar *et al*. 2015). Higher air temperature results in higher abnormal spermatozoa, decreased embryo quality, lower live spermatozoa and higher DNA fragmentation index (Valeanu *et al*. 2015). Keeping in mind the above facts, present study was carried out to evaluate the effect of AX supplementation to crossbred bulls on their semen quality, lipid peroxidation and the expression of mitochondrial activity related genes in semen during summer season under tropical climatic conditions.

MATERIALS AND METHODS

Ten healthy Karan Fries (crossbred) bulls were selected and equally divided into 2 groups i.e. control and treatment (Astaxanthin supplemented) and maintained at Animal Breeding Research Centre of the institute. The environmental parameters recorded during the experimental period are presented in Table 1. The temperature humidity index (THI) was calculated using following equation of McDowell (1972):

THI = 0.72 (C_{db} + C_{wb}) + 40.6, where C_{db} and C_{wb} are dry and wet bulb temperature in °C.

Bulls were given a bath at least 40 min before semen collection. Ejaculates were collected at a weekly interval from April to August using artificial vagina (42–45°C) at early in the morning. Immediately after collection, samples were placed in a water bath (37°C) for semen analysis, viz. volume, mass motility, progressive motility, hypo-osmotic swelling test (HOST), live sperm count, acrosome integrity, sperm concentration and sperm abnormalities.

Bulls were offered green and dry roughages *ad lib.* as per the availability and concentrate mixture @ 2.5 kg/day/animal. Concentrate mixture consisted of 28% maize, 10% ground nut cake, 13% mustard cake, 15% wheat bran, 11% rice polish, 15% soyabean deoiled, 5% bajra, 2% mineral mixture and 1% salt with 16% CP and 70% TDN. The supplementation of natural AX @ 0.25 mg/kg body weight/day (Lignell and Inborr 2002) was started from April and continued to August. AX powder was mixed properly with 2.5 kg of concentrate mixture and offered to bulls as per their body weight. Water was made available to the animals round the clock.

Semen analysis: Ejaculates were collected in a graduated centrifuge tube. A drop of fresh semen was placed on a preheated (37°C) glass slide and observed under a phase contrast microscope (Nikon eclipse E600, Tokyo, Japan) at low magnification (10×) with a coverslip and graded on the basis of wave movement i.e. mass motility as 0 (Waves not present, sperm cells immotile), + (1 = Waves not present, sperm cells motile), ++ (2 = Barely distinguishable waves in motion), +++ (3 = Waves apparent, moderate motion) and ++++ (4= Dark distinct waves in rapid motion). Progressive motility was assessed by diluting the neat semen with egg yolk medium (1:10), 4-5 µl of diluted semen was placed on a preheated glass slide (37°C) and observed under a microscope (40×). Hypo-osmotic swelling test (HOST) and live and dead spermatozoa (eosin-nigrosin stain) were assessed using standard methods. The eosin-nigrosin stain was also used for counting sperm abnormalities, viz. major abnormalities (Proximal cytoplasmic droplet, pyriform heads, folded/coiled tails and middle piece defects) and minor abnormalities (Distal cytoplasmic droplets, tailless normal heads, simple bend, terminally coiled tail, narrow and small heads). The spermatozoa having either completely or partially stained pink (eosin) heads were considered as dead and the unstained considered as live sperm. Acrosome integrity was assessed by using Giemsa stain. The attached acrosome showed purple colour and detached acrosome without purple coloured on heads of the spermatozoa. Sperm concentration was determined by haemocytometer (Neubauer improved, Marienfeld).

Total antioxidant capacity (TAC) and malondialdehyde (MDA) assay: Two milliliters of semen sample was taken just after collection in an Eppendorf tube and centrifuge at 10,000 rpm for 10 min at 4°C. The supernatant (seminal plasma) was centrifuged again at 10,000 rpm for 5 min at 4°C; finally, the supernatant was collected and kept at –20°C until assay was carried out. Total antioxidant capacity (TAC: Cat. No. MBS748686) was carried out using bovine ELISA kit as per the manufacturer's protocol. The sensitivity of the assay kit was 1 ng/ml. Malondialdehyde (MDA) concentration was determined by Quantichrom™ TBARS assay kit (DTBA-100, USA) as per the manufacturer's protocol. The optical density was recorded using TECAN infinite PRO200 ELISA reader (Tecan Asia

Table 1. Environmental parameters recorded during the experimental period

Variable	April	May	June	July	August
Maximum temperature (°C)	34.1±0.63	37.6±0.58	40.9±0.62	34.5±0.48	34.4±0.20
Minimum temperature (°C)	16.1±0.42	21.7±0.29	26.1±0.37	26.7±0.27	25.9±0.18
Dry bulb temperature (°C)	19.8±0.53 AM	24.8±0.37 AM	28.6±0.34 AM	28.1±0.32 AM	27.2±0.23 AM
	33.8±0.65 PM	36.8±0.57 PM	39.5±0.69 PM	33.0±0.61 PM	33.4±0.30 PM
Wet bulb temperature (°C)	19.6±3.35 AM	20.4±0.26 AM	24.0±0.40 AM	26.3±0.20 AM	25.7±0.20 AM
	19.4±0.22 PM	22.7±0.30 PM	25.7±0.34 PM	27.0±0.26 PM	27.8±0.15 PM
Relative humidity (%)	69.6±2.43 AM	66.0±2.20 AM	67.0±2.63 AM	82.2±1.10 AM	88.8±1.00 AM
	22.1±1.73 PM	27.6±1.94 PM	32.2±2.74 PM	62.5±2.29 PM	63.7±1.28 PM
Temperature humidity	69.0±2.42 AM	73.1±0.39 AM	78.5±0.45 AM	79.8±0.36 AM	78.7±0.30 AM
index (THI)	60.6±0.42 PM	65.8±0.61 PM	70.8±0.91 PM	76.7±0.44 PM	78.1±0.28 PM
Sunshine (hours)	9.7±0.40	9.2±0.48	7.3±0.50	6.4±0.69	7.2±0.6

Pvt. Ltd, Singapore) at 450 and 535 nm for TAC and MDA, respectively. The Intra and inter-assay coefficient variation were <10%.

Primer designing : The corresponding mRNA sequences of selected genes from available bovine species were retrieved from NCBI database (www.ncbi/nlm/nih.co.in). The primer sequences are presented in Table 2. The melting temperatures (Tm), the formation of the hairpin and internal secondary structures were checked by Primer Stats (http:// www.bioinformatics.org/sms2 pcr_primer_ stats. html). The candidate genes [(*succinate dehydrogenase* (SDH), *citrate synthase* (CS) and *mitochondrial transcription factor A* (TFAM)] were selected which indicates the respiratory activity, number or volume and transcription activities of mitochondria.

Separation of motile spermatozoa: Swim up technique was used to eliminate the damaged spermatozoa, contaminated somatic cells and non-motile spermatozoa. Briefly, 0.5 ml of semen was placed at the bottom of 15 ml centrifuge tube containing modified TALP (Tyrode's albumin lactate pyruvate) medium in 1:5 ratio. The tubes were kept at 45°C angles in a CO_2 incubator maintained at 37°C, 5% CO_2 with 80–90% relative humidity for 60 min. The motile sperms were collected (after incubation) by aspiring 3/4th top fraction of the medium. An equal amount of fresh TALP medium was added and centrifuged for 10 min at 1500–1800 rpm (REMI-4C). Supernatant was discarded; 1ml of TRIzol® LS Reagent (Ambion by life technology, USA) was added to the pellet for total RNA isolation.

Total RNA extraction, RNA quantification, purity and semi-quantitative PCR: Total RNA was isolated using TRIzol® LS reagent with minor modifications. For each sample, about 200 ng of total RNA was used for cDNA synthesis using Revert Aid First strand cDNA synthesis kit (Fermentas, USA) by reverse transcription-polymerase chain reaction (RT-PCR) according to the manufacturer's protocol. Briefly, the mixture of RNA and oligo (dt) primer, 5× reaction buffer, Ribolock RNase inhibitor (20 U/µl), 10 mM dNTP mix and revertAid M-MuLVRT (200 U/µl) were added to a 0.2 ml sterile tube and made it to 20 µl by adding nuclease-free water. The RT-PCR was carried out at 65°C for 5 min, 42°C for 60 min and 70°C for 5 min in a thermocycler (AB Applied Biosystem). The cDNA product was diluted into 1:1 dilution and stored at –20°C to perform downstream PCR amplification and qPCR.

Semi-quantitative PCR (qPCR, Applied Biosystems® 7500 Real-Time PCR) was used to analyze the relative expression of candidate genes in ejaculated spermatozoa. The annealing temperatures for all the primers were evaluated through gradient PCR (Bio-Rad, USA), amplification of candidate genes were confirmed by observing the product size (using 2.5% agarose) under a Gel documentation system (Bio-Rad). The Semi-quantitative PCR reaction was carried out using Maxima SYBR green real-time PCR (qPCR) mAXer mix (10 µl) along with forward and reverse primers (1 µl, 10 pmol), nuclease free sterile water (7 µl) and template (1 µl). Negative controls were run in each PCR assay without template (cDNA). The PCR product of candidate genes was confirmed (2.5% agarose) by observing in Gel documentation system (Fig. 1). The qPCR program consisted of initial heating at 50°C for 2 min followed by 95°C for 10 min, annealing (Table 2) for 60 sec, and amplified for 40 cycles. The final extension at 72°C incubation was continued for a further 10 min.

Ethical permission: The experiment was approved by the Institutional Animal Ethics Committee (IAEC) constituted as per the article number 13 of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) rules laid down by the Government of India.

Statistical analysis: The data obtained for semen quality parameters, semen MDA and TAC levels between the two groups were compared using two sample PROC T TEST of the SAS software, Version (9.1) (SAS Institute Inc., Cary, NC, USA). The relative expression of candidate genes (SDH, CS, and TFAM) was calculated by comparing the expression level of reference gene β-Actin as per the method of Livak and Scmittgen (2001). The level of expression of SDH, CS and TFAM between the groups were also compared using two sample PROC T TEST of the SAS software. Graphs were plotted using Prism 5.

RESULTS AND DISCUSSION

Semen quality: The availability of good quality semen is essential throughout the years for the sustainable dairy development. However, semen quality found to compromise in Karan Fries bulls during summer season under tropical climatic conditions (Soren *et al*. 2016a). Higher sperm

Table 2. Real-time primers for candidate and housekeeping genes

Gene	Primer sequence	Annealing Temp. (ºC)	Fragment size (bp)	Reference
SDH	F-AAATGCCCGGCTTTACTTGC R-GAGGAGCCAGCAGTGGTATT	56.8	181	Soren *et al*. (2017)
CS	F-CCTGATGAGGGCATCCGTTT R-CCACTCCTGTGAGAGCCAAG	56.8	165	Soren *et al*. (2017)
TFAM	F-AGGAAGCTAGGGATGGCACA R-CTTCTCGTCCAACTTCCATCA	56.8	174	Soren *et al*. (2017)
b-ACTIN	F- AGGCATCCTGACCCTCAAGTA R- GCTCGTTGTAGAAGGTGTGGT	52–60	95	Soren *et al*. (2017)

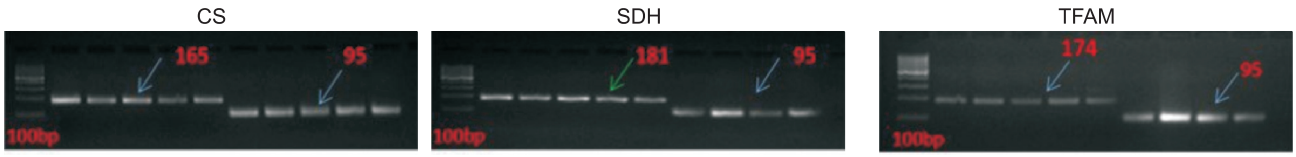

Fig. 1. *Citrate synthase* (CS), *succinate dehydrogenase* (SDH) and *mitochondrial transcription factor A* (TFAM) PCR product size in Gel-documentation system.

abnormalities, sperm DNA damage with reduced fertility were observed during summer season, reported by several authors (Nichi *et al*. 2006, Valeanu *et al*. 2015). The supplementation of astaxanthin showed a positive (P<0.05) effect on the semen quality of Karan Fries bulls during summer season (hot and humid) (Fig. 2 A, B, C, D and F). The major abnormalities (%) decreased (P≤0.05) (Fig 2E), however, no significant (P<0.05) difference was observed between the groups in mass motility, live sperm count, HOST and minor abnormalities in astaxanthin supplemented group of bulls (Table 3). Oral supplementation of AX improved the semen quality, fertilization and conception rate in human (Comhaire *et al*. 2005). Ameliorative effect of AX on semen quality was noticed in the present study during summer (heat stress) season in Karan Fries bulls. The percentage of progressive motility and acrosome integrity were improved, the sperm major abnormalities decreased during the summer season in AX supplemented bulls. Similarly, Tripathi and Jena (2008) also showed improvement in semen quality of mice supplemented with AX. Supplementation of AX might be one of the improvement strategies of semen quality suggested by Lignell and Inborr (2000).

Total antioxidant capacity and lipid peroxidation in semen plasma: The semen malondialdehyde (MDA) concentration was markedly decreased (P<0.01) in AX supplemented bulls during summer (Table 4). The higher (P<0.05) semen TAC was also observed during July and August in AX supplemented bulls (Table 4). The higher concentration of seminal antioxidant enzymes was reported previously during summer season than that of winter season (Soren *et al*. 2016b). Impairment of mitochondrial activity, structural damage to biomolecules (DNA, lipids, carbohydrates and proteins) and other cellular components were showed in heat stress bull's semen raised under tropical climatic conditions (Nichi *et al*. 2006). Astaxanthin demonstrated to be a potent antioxidant, capable of enhancing the mitochondrial activity in culture cell line (Wolf *et al*. 2010). AX might prevent the lipid peroxidation and reduced the oxidative stress by either accepting or donating electrons without being destroyed or becoming a pro-oxidant in the process (Guerin *et al.* 2003, Olaizola and Huntley 2003, O'Connor and O'Brien 1998). Supplementation of AX also reported to improve the progressive motility and decreased the lipid peroxidation rate in semen of Holstein bulls (Farzan *et al*. 2014).

Gene expression: The relative expression (mRNA) of *succinate dehydrogenase* (SDH), *citrate synthase* (CS) and *mitochondrial transcription factor A* (TFAM) was up regulated (P<0.01) in spermatozoa of AX supplemented bulls as compared to non-supplemented bulls during different months of summer (Fig. 3A, B, C). Several studies reported the relationship of mitochondrial respiratory enzyme activity with sperm motility (Ruiz-Pesini *et al*. 2000) and also with male fertility (Cunmins *et al*. 1994, St John *et al*. 1997). The mitochondrial respiratory complex activity reflects the electron transfer capacity, whereas citrate synthase (CS) considered as a reliable marker of the number and or volume of mitochondria (Di Donato *et al*. 1993). The relative expression of SDH, CS and TFAM gene was found to be lowered in ejaculated spermatozoa collected during summer season than winter (Soren *et al*. 2018). The heat shock protein genes were higher in ejaculated spermatozoa during summer than winter (Soren *et al*. 2018). The variation of physio-chemical properties of semen during different season showed the significant impact of heat stress on semen quality. Supplementation of AX might augment the mitochondrial function under heat stress during summer season. Ikeuchi *et al*. (2005) reported that the higher expression of mitochondrial complex enzymes and mtDNA

Table 3. Average values of mass motility, live sperm count, HOST and minor sperm abnormalities in control and supplemented Karan Fries bulls during summer

Parameter	April		May		June		July		August	
	C	T	C	T	C	T	C	T	C	T
Mass motility	2.85± 0.07	2.47± 0.19	2.55± 0.12	2.55± 0.20	2.37± 0.10	2.55± 0.20	2.42± 0.09	2.57± 0.99	2.30± 0.09	2.37± 0.22
Live sperm count (%)	67.53± 0.95	68.78± 2.88	68.17± 0.98	68.13± 2.23	66.52± 0.98	67.70± 2.23	63.56± 0.80	65.65± 2.42	62.39± 0.93	63.86± 2.42
HOST (%)	66.04± 1.46	65.59± 1.58	64.72± 0.76	63.16± 1.01	62.3 6± 1.06	62.41± 0.80	59.92± 0.97	61.93± 1.11	59.58± 0.60	61.49± 1.09
Minor abnormalities (%)	8.22± 0.43	7.86± 0.42	7.96± 0.40	8.02± 0.40	8.38± 0.18	8.10± 0.38	8.04± 0.36	7.96± 0.31	8.24± 0.26	8.36± 0.36

Table 4. Effect of astaxanthin supplementation on seminal total antioxidant capacity and malondialdehyde concentration during summer

Parameter	April		May		June		July		August	
	C	T	C	T	C	T	C	T	C	T
TAC (ng/ml)	90.92± 2.94	91.38± 2.30	93.56± 2.42	93.42± 1.90	96.77± 3.04	103.2± 2.27	100.7± 3.51	112.2**± 2.32	104.3± 3.68	118.4**± 1.73
MDA (μM)	19.16± 0.77	17.89± 0.84	22.91± 1.06	20.23*± 0.56	29.05± 1.16	24.52**± 0.85	33.87± 1.24	26.88**± 0.64	36.06±.45**± 0.9928	28.45*± 0.76

Values with *(P <0.05) and **(P <0.01) within the row during month differed significantly.

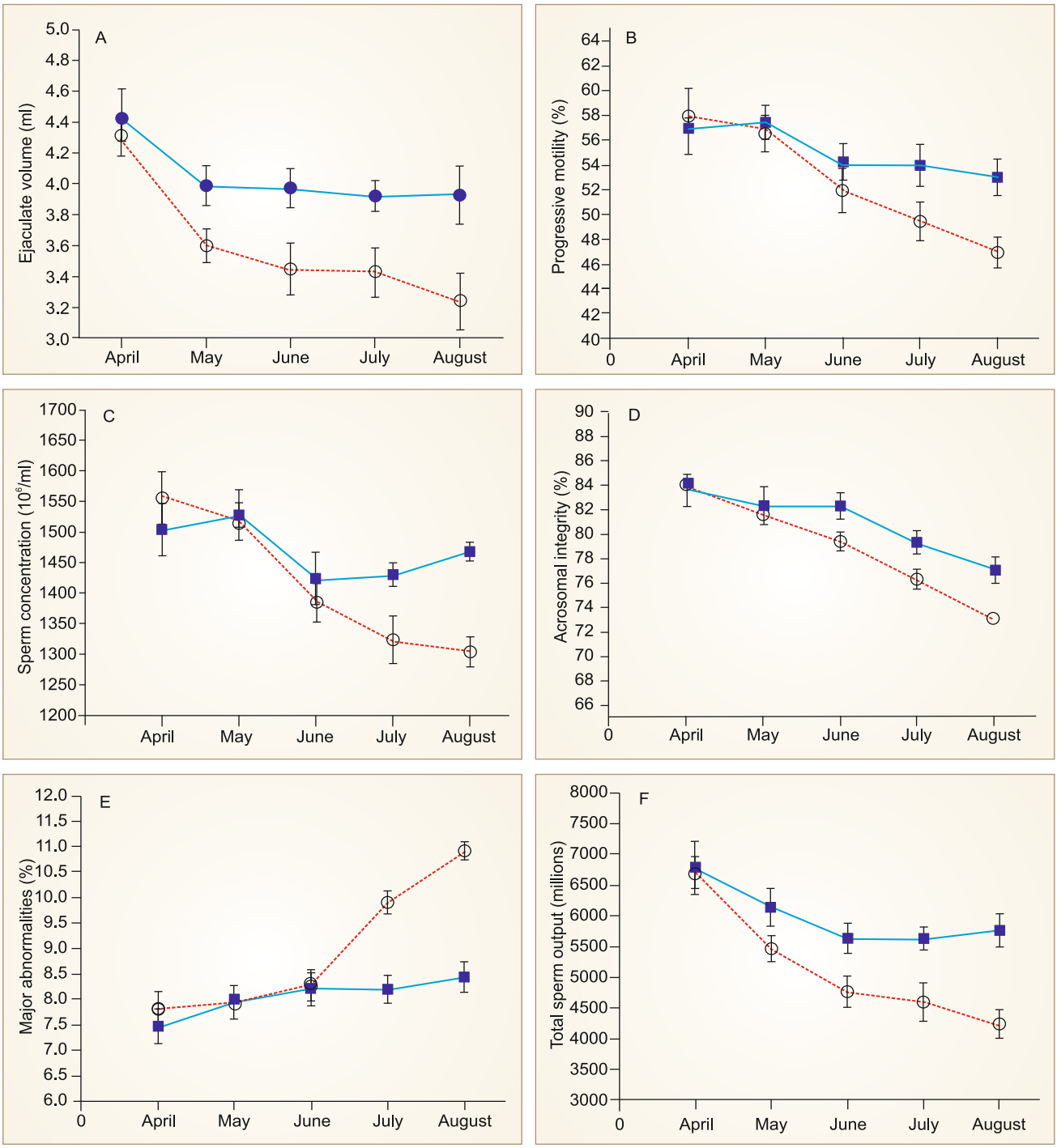

Fig. 2. Effect of AX on semen evaluation parameters. **A**. Ejaculate volume (ml). **B**. Progressive motility (%). **C**. Sperm concentration (10^6/ml). **D**. Acrosomal integrity (%). **E**. Major abnormalities (%). **F**. Total sperm output (millions).

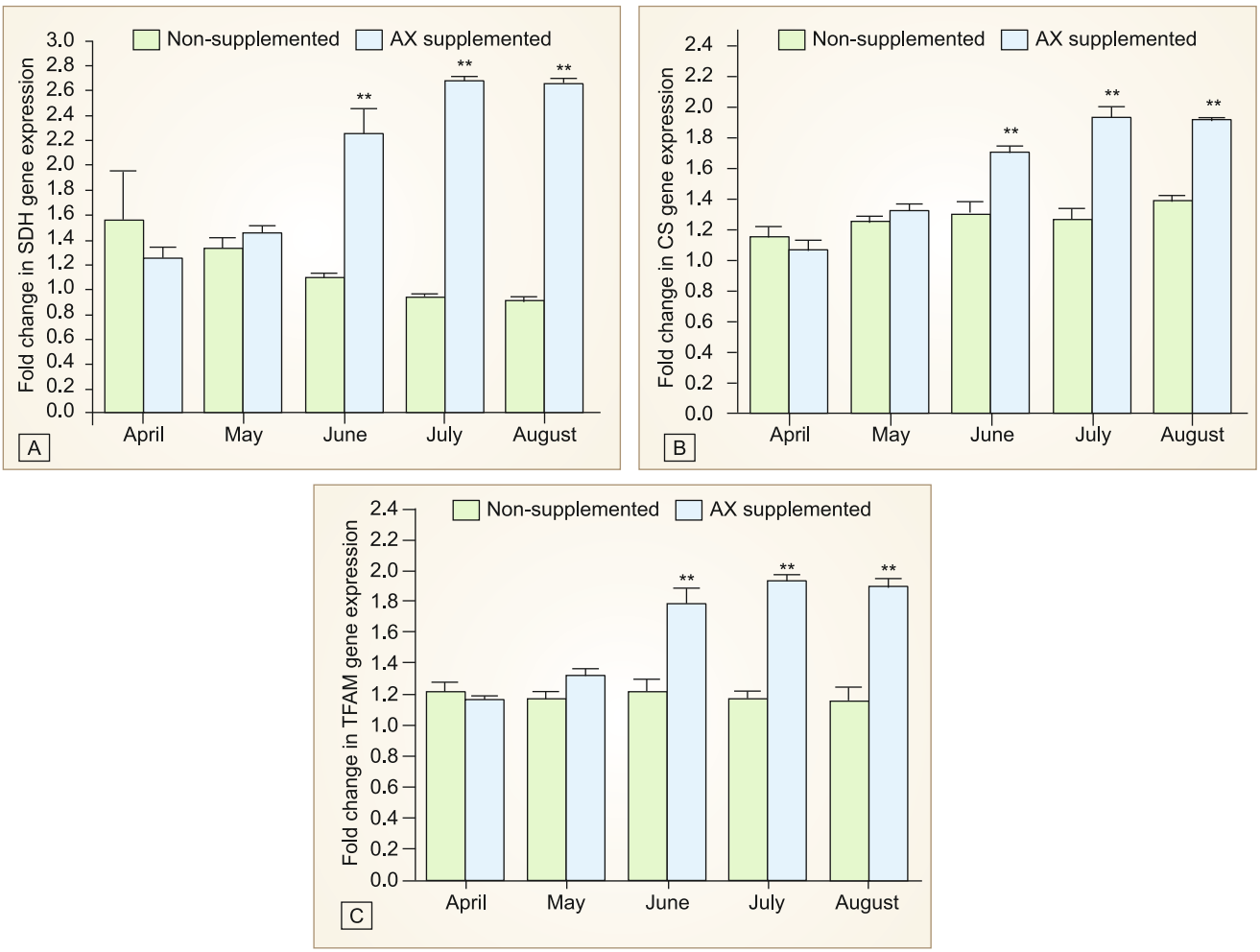

Fig. 3. Effect of AX on relative mRNA expression of succinate dehydrogenase (**A**), citrate synthase (**B**) and mitochondrial transcription factor A (**C**) in spermatozoa of Karan Fries bulls during summer.

copy indicates enhancement of mitochondrial function. The study of Kuroki *et al*. (2013) found co-localize of AX with mitochondria in heat stress embryos, which reveal the ameliorative effect of AX on heat stress blastocyst development.

The poor semen quality and lower fertility of European bulls under tropical conditions hypothesised to be oxidative stress or insufficient defensive mechanism to combat the oxidative stress (Nichi *et al*. 2006). The cell organelles, mitochondria are more on the risk of oxidative stress. The generation of free radicals via electron transport chain or mitochondrial respiratory activity is more common and the mitochondrial membrane is high risk of free radical attack. Overproduction of free radicals limits the mitochondrial function and supply of energy (ATP) reduced for sperm motility resulted poor motility.

The ameliorative effect of astaxanthin on semen quality of Karan Fries bulls was observed in the present study against heat stress. Decreased concentration of malondialdehyde (MDA) and the higher expression of (mRNA) of *succinate dehydrogenase* (SDH), *citrate synthase* (CS) and *mitochondrial transcription factor A* (TFAM) in spermatozoa of AX supplemented bulls indicate the positive effect of AX on sperm mitochondrial respiratory activity.

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