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Original Article

Dietary *Chlorella vulgaris* Supplementation Improves Reproductive Index of Female Rabbits and Protect their Progeny against Oxidative Stress

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ABSTRACT

Background: Oxidative stress negatively impacts pregnancy and its outcomes; hence, its avoidance through gestational antioxidant intakes is a desired nutrition-reproduction practices. **Materials and Methods:** Female rabbits (*n*=40) were divided into 5 groups and supplemented with 0, 200, 300, 400, and 500 mg *Chlorella vulgaris* biomass per kilogram body weight daily, respectively throughout the gestation period. Upon kindling, kits of the female rabbits (*n*=75) were randomly selected and monitored from birth till 120 days old. The reproductive performance of the female rabbits was evaluated by computation of their reproductive index, while birth weight, growth rate, oestrogen and testosterone concentrations, oxidative stress biomarkers, and expression of selected functional genes of the progeny were determined. **Results:** The gestational intake of *Chlorella vulgaris* increased reproductive index of the female rabbits ($p < 0.05$), and it also increases the growth and oxidative stress protection status of the rabbit progeny ($p < 0.05$). Selected functional genes, including *Gstp1*, *Cyp1a1*, *Ar*, *Ghr*, *Il2*, and *Il6* assessed in the progeny of the supplemented groups were significantly upregulated ($p < 0.05$). **Conclusion:** It was concluded from these results that gestational *Chlorella vulgaris* biomass intake improved reproductive index of the female rabbits, increased growth and oxidative stress protection in their progeny.

Keywords: New Zealand Rabbit, Oxidative Stress Biomarkers, Reproductive Activity, Relative Gene Expression.

INTRODUCTION

Reproduction is the process of bringing forth an offspring and it is synonymous with breeding, procreation, propagation, multiplication, and measuring of the biological productivity of animals or humans.[1] However, reproduction is inherently associated with generation of free radicals and reduced antioxidant defense which makes oxidative stress a subject matter of interest in reproduction science.[2] Meanwhile, oxidative stress could be both beneficial or damaging to reproduction, depending on the stages of reproduction of interest; for instance, during the gestation period, oxidative stress is a mechanism employed by pregnant mammals for the elimination of pathogenic agents and maintenance of a disease-free reproductive tract but fetal exposure to free radical is capable of causing havoc to the developing fetus.[3]

The fetal exposure to an imbalance prooxidants and antioxidants regime has been reported as a possible *in-utero* physiological disturbances capable of causing either temporal or permanent changes in fetal development such as the compromising of neonatal growth, and adulthood fertility.[4] Furthermore, the inherent generation of free radicals via multiple routes such as the maternal leucocytes, endothelium, and placental oxidative phosphorylation during gestation period are another damaging effects of oxidative stress associated with reproduction which could comprise both the dam and fetal well-being.[5] Hence, the maintenance of a balance between free radicals and antioxidants is warranted for the benefits of both the dam and the fetus before and after birth.

Studies in the past have explored maternal intakes of Vitamins C and E as antioxidants suitable for protective purposes, but they were reported not to provide sufficient protection required against gestational oxidative stress and its complexities.[6,7] Therefore, there is a need for continuous search of alternative antioxidants capable of protecting against gestational oxidative stress for promotion of both the maternal and fetal well-being as well as the prevention of adulthood performance dysfunctions associated with *in-utero* oxidative stress exposure.[8]

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Chlorella vulgaris is a micro-alga with the desirable antioxidant properties due to its reported capacity to inhibit gestational oxidative stress in rabbits and other animals.[9,10] It was also reported in an earlier study that the supplementation of *C. vulgaris* in pregnant and lactating sheep enhanced activity of antioxidant enzymes and also increase growth of their lambs.[11] Therefore, this study explored the effect of the micro-alga *Chlorella vulgaris* intake as an antioxidant supplement on oxidative stress protection in pregnant rabbits and programming of their progeny for higher birth weight, faster postnatal growth rates, and imprinted molecular protection against oxidative stress.

MATERIALS AND METHODS

The Rabbit does and their Management

Forty matured New Zealand White rabbits does (average weight = 2092.03±20.74 g) were distributed into five experimental groups (n = 8 per group), designated as control, T1, T2, T3, and T4, respectively. The rabbit does were acclimatized for two weeks, after which they were naturally serviced by active bucks, then housed individually, supplied with commercial rabbit feed (Table 1), and provided with water *ad-libitum*. In addition to the basal feed, the rabbit does were supplemented with 0, 200, 300, 400, and 500 mg *Chlorella vulgaris* biomass per kg body weight as an antioxidant, respectively, per each rabbit doe in the control, T1, T2, T3, and T4 groups, from gestation day 0 till kindling.

Computation of Reproductive Indices of the Rabbit does

The reproductive index (RI) was used as a measure of the overall reproductive performance of rabbit does from conception to kindling. This was computed for each rabbit doe used in the experiment with reproductive parameters including litter size, kindling rates, and viability of the rabbit kits kindled by the rabbit does using the formula:[12]

RI = Litter size × Kindling rate × Viability rate --- equation i

Determination of kit birth weight and growth performances

The individual birth weight of the rabbit kits was recorded immediately after birth using a laboratory sensitive balance (Atom Scales, India). The weighing procedure involves placing of the kit individually on the balance for about 60 sec while the record of the body weight was obtained. The kits were standardized as 5 kits per doe throughout the 21 day lactation period and at weaning, there was a random selection of 15 male kits for each of the groups which were observed for growth performances. The selected male kits were differentiated from their female counterparts using the presence of a protruding circular genital organ in the male against the flatter slit genital in the female kits.[13] The records of daily feed intakes, weekly body weight changes, and feed conversion ratio of the male kits were recorded from day 21 of their selection till 120 days old when they were sacrificed for blood and livers collection.

Table 1: Chemical and nutritional composition of the commercial basal feed fed to the rabbits.

Parameters	Amount	Unit
Dry matter	90.89	% dry matter
Crude protein	18.55	% of dry matter
Total ash	7.90	% of dry matter
Metabolizable energy	2700	kcal/kg
Crude fibre	9.73	% of dry matter
Ether extract	2.99	% of dry matter

Each rabbit was provided with 20 g of *Pennisetum purpureum* daily as part of the basal feed

Blood and Liver Collection, Processing, and Storage for Biochemical Analyses

***Blood collection and storage*:** For the rabbit does, 2 mL of blood sample was obtained through the ear veins on the last week of the gestation, for the kits, they were subjected to cervical dislocation followed by a sharp cut through the jugular vein for blood collection. The blood was collected into vacutainers and kept at room temperature for 15 to 30 min to clot after which they were centrifuged at 2000 rpm for 10 min (Remi Centrifuge, India). The harvested serum from the centrifuged blood samples were collected into test bottles then kept at -80°C ahead of downstream analyses.[10,14,15]

***Liver collection and preparation of homogenates*:** The liver of the sacrificed rabbits was removed, snap-frozen in liquid nitrogen then stored at -80°C for downstream analyses. For the preparation of the liver homogenates, the snap-frozen liver samples were thawed overnight at 4°C, homogenized in a buffer solution containing Ethylene Diamine Tetra Acetic acid (EDTA), (DDT), and 50 mM Tris HCl solution. The homogenate contained 100 mg of the liver tissue in 1 mL buffer homogenized in a handheld homogenizer, then centrifuged for supernatants collection which were also stored at -80°C for downstream analyses.[16,17]

***Determination of the protein concentration of the serum and liver homogenates*:** For the downstream biochemical evaluation, the protein concentration of the serum and liver homogenate samples were determined using the Bicinchoninic Acid (BCA) protein assay kit (G-Biosciences, USA) according to the manufacturer's protocol. In summary, the procedures involved a preparation of protein standards of known concentration using Bovine Serum Albumin (BSA) through serial dilution; then 25 µL of each sample and the standard were reacted with 200 µL BCA and incubated at 37°C (Labnet Inc., USA). After the incubation, the absorbance of both the samples and standards were determined at 562 nM using a Thermo Multiskan GO microplate reader (Thermo Fisher Scientific, Finland).

***Quantification of the kit's serum oestrogen and testosterone concentration*:** The serum concentrations of oestrogen and testosterone of the rabbit kits were determined using commercial estradiol and testosterone ELISA kits according to the manufacturer protocols (Calibiotech, Inc., USA). For oestrogen concentration, 25 µL of each sample and standards were added with 100 µL working solutions comprising anti-estradiol polyclonal antibody then incubated at 25°C for 60 min in coated wells. After incubation, the wells were washed three times with wash buffer and blotted using an absorbance paper. Then the washed wells were then incubated with 100 µL of the 3,3′,5,5′-Tetramethylbenzidine (TMB) reagents for 30 min at 25°C then the reaction was stopped with 50 µL stop solution provided in the kit, and absorbance readings were observed at 450 nm in a microplate reader and the readings obtained were used for computation of the concentration of estradiol in pg/mL.

For the testosterone concentration, 50 µL of each sample and standard were added with 100 µL working solution containing conjugated testosterone enzymes and 100 µL biotin reagent then incubated at 25°C for 60 min in coated wells. After incubation, the wells were washed three times with wash buffer and blotted with absorbance paper. The washed wells were then incubated with 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) reagents for 30 min at 25°C and the reaction was stopped with 50 µL stop solution provided in the kit. The absorbance readings were observed at 450 nm in a microplate reader and the concentration of testosterone was determined in ng/mL using the reading obtained.

***Determination and quantification of lipid peroxidation biomarker*:** Lipid peroxidation product malondialdehyde concentration was determined in the serum and liver samples. This involved preparation of a reaction mixture comprising 0.5 mL of serum, 0.2 mL of 8% sodium *n*-dodecyl sulfate, 1 mL of 20 % acetic acid, and 1 mL of 0.8 % 2-thiobarbituric acid (TBA) was prepared; and boiled for 1 hr at 95°C in a water bath (AMETEK Instruments, India). The reaction was terminated by cooling the mixture on ice for 10 min then 1 mL *n*-butanol and pyridine (15:1; v/v) was added to each reaction and vortexed briefly then centrifuge at 4000 rpm for 10 min. Organic supernatant layers were obtained from the mixtures after centrifugation and absorbance readings were measured at 532 nm in a 96-wells microplate reader.[17]

Determination of serum and liver protein carbonylation biomarker: The concentration of protein carbonyl in the serum and liver samples were determined following the protocol procedure involving labelling protein carbonyl with 2,4-dinitrophenylhydrazine.[18] This entails sampling of the serum and tissue samples for determining the quantity of carbonylated protein concentration per 1 ml of the serum sample and per 1 mg of the liver which was calculated using the protein carbonyl molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

***Determination of serum and liver total antioxidant capacity*:** The overall antioxidant capacities of the serum and liver samples were determined using the protocol described by Benzie and Strain (1996). The assay principle is based on the reduction of iron (III) to iron (II) at low pH. In this study, a freshly prepared Ferric Reducing Antioxidant Power (FRAP) working assay containing 25 mL sodium acetate buffer (300 mM, pH 3.6), 2.5 mL 10 mM 2,4,6-Tri(2 pyridyl)-s-trizine prepared in 40 mM HCl and 2.5 mL Ferric chloride (20 mM prepared in distilled water).[19]

***Determination of superoxide dismutase activities*:** The activities of the superoxide dismutase enzyme in the serum and liver samples. Briefly, it is a chemical assay comprised of 50 mM Tris cacodylate buffer, 1 mM DEPTA, and 20 mM Pyrogallol solution prepared in 10 mM HCl. The reaction time was 180 sec and at the end of spectrometer absorbance readings, inhibition of pyrogallol auto-oxidation and activity of the superoxide dismutase enzymes in the samples were calculated.

***Determination of serum and liver catalase activities*:** The activity of catalase as an antioxidant enzyme was determined using the decomposition of hydrogen peroxide (H_2O_2) into H_2O and O_2 in the presence of catalase in the samples (EC 1.11.1.6). The assay was composed of 50 mM potassium phosphate buffer (pH-7.0) prepared from di-potassium hydrogen phosphate, potassium dihydrogen phosphate, and 100 µM hydrogen peroxide. The reaction mixtures were read at 240 nm and the absorbance changes were noted at every 30 sec during 180 sec reaction time for each sample.[20]

***Determination of serum and liver glutathione concentration*:** The concentration of reduced glutathione was determined using a chemical assay comprising 20 % trichloroacetic acid, 200 mM phosphate buffer (pH 8.0), and 5 mM 5,5´- diothiobis (2-nitrobenzoic acid). This chemical assay was incubated with each sample at room temperature for 10 min, and reduced glutathione standards were prepared alongside the test samples. The absorbance of both the standards and the samples was read at 412 nm in a microplate reader (Thermo Fisher Scientific, Finland). The concentration of reduced glutathione in each sample was calculated in µmol of glutathione (GSH) per mL of serum and per g of the liver samples.[16]

***Determination of the serum biochemical profile*:** Serum biochemical parameters include alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), creatinine, alkaline phosphatase (ALP), bilirubin, and urea concentration. These parameters were determined using commercial kits (Proton Biologicals, India). The manufacturer procedures were followed in endpoint reactions measured by spectrophotometer for each of the biochemical parameters.[10]

Quantification of Relative Expression of Selected Functional Genes in the Rabbit Kits

Some functional genes expressions were quantified, the selection of the genes was based on their roles as an antioxidant (*Gstp1*), detoxifiers of free radicals (*Cyp1a1*), promoter of male reproductive system development (*Ar*), regulation of growth rates (*Ghr*), and regulation of innate and adaptive immune functions (*Il2* and *Il6*). The oligonucleotide primers used for the gene expression studies were designed using the database of the National Centre for Biotechnology Information (NCBI) while glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal reference control gene (Table 2). The procedures for the quantification of relative expression of the selected target genes were determined as reported earlier.[17] The procedure involves isolation of total RNA from the liver samples of the rabbits, followed by synthesizing of cDNA from the RNA for real-time PCR quantification. The respective cDNA templates of each liver sample, 20 ng/µL samples per reaction volume of real-time PCR quantification was carried out using the StepOne Real-Time PCR system (Applied Biosystem, USA). Takara SYBR® premix was used for the comparative quantification of gene expression (Takara Bio, Japan). The quantification reaction for the gene expression followed Takara SYBR® premix protocol for Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System and StepOne Real-Time PCR System. The reactions ran first for 1 min at 95°C; the second step was denaturation at 95°C which ran for 5 sec then annealing ran at 61°C for 1 min continuously for 40 cycles.

Statistical Analysis

The data obtained were subjected to a one-way analysis of variance using SPSS version 20.0 (IBM, USA). Significant means were determined at $p < 0.05$ while means separation was done using the Duncan test *post-hoc* tools in the software.

RESULTS

The Reproductive Performances of the Rabbit does

There was a significant difference observed in the gestation length of the rabbit does ($p < 0.05$). The mean gestation length was 31.79±0.31 days ($p = 0.01$), while the minimum and maximum gestation lengths were 30.42±0.20 and 33.00±1.03 days, respectively. There was also a significant difference observed in the kindling rate of the rabbit does ($p < 0.05$). The mean kindling rate was 76.46±2.98 % ($p = 0.001$), while the minimum and maximum kindling rates were 57.14±0.16 %, and 100.00±0.00 %, respectively. Furthermore, there was a significant difference observed in the reproductive index of the rabbit does; the mean reproductive index was 3.82±0.43 ($p = 0.01$), while the minimum and maximum reproductive indexes were 2.04±0.78, and 6.14±0.59, respectively (Table 3).

Kits Birth Weight, Post-weaning Growth, and Hormones Concentrations

The gestational intakes of *Chlorella vulgaris* biomass led to a significant difference observed in the birth weight, and post-weaning body weight gains of the rabbit kits (Table 4). The supplemented group kindled rabbit kits with higher birth weight compared with the control group (50.25±1.64 vs 34.89±3.40 g, $p = 0.04$). There was better feed utilization efficiency by kits of the supplemented group compared with the control group despite a reduction in daily feed intake (45.15±2.22 vs 63.85±2.25 g, $p = 0.03$) leading to the improved feed conversion ratio of the kits of the treatment groups compared with the control (1.63±0.10 vs 2.60±0.10,

Table 2: The selected functional target genes and their oligonucleotide primers used for evaluation of gene expression in the rabbit kits.

S/N	Gene Name	Primer type	Primer Sequence (5′ to 3′)	Primers Length (bp)	Amplicon size	Accession No
1	Growth hormone receptor (GHR), mRNA	F	TTCACAGCCTTTACCCAGACA	21	200	NM_001082636.1
		R	CTGGACTACTTGGAGGGAAATAA	23		
2	Interleukin 6 (IL6), mRNA	F	GGAGGACTCCAACACCAAGG	20	230	NM_001082064.2
		R	AGGTCTCATTATTCACCGCCG	21		
3	Interleukin 1 alpha (IL1A), mRNA	F	GCACTTGAGTCGGCAAAGAAAT	22	228	NM_001101684.1
		R	GGAAGGTGAGGTTGGGTGAC	20		
4	Interleukin 2 (IL2), transcript variant 1, mRNA	F	TGCACTAACTCTTGCACTCCT	21	249	NM_001163180.1
		R	AGCATCCTGGAAAGTTTGGA	20		
5	Androgen receptor (AR), mRNA	F	GACTCTGTGCAGCCTATTGC	20	208	NM_001195724.1
		R	GTGCGGTGGAGTTAGGGAAA	20		
6	Nuclear receptor subfamily 3 group C member 1 (NR3C1), mRNA	F	AAGGGCAGTGAAAGGACAGC	20	243	NM_001082147.1
		R	TGTGGTAATGCTGCAGGAACT	21		
7	Nuclear factor kappa B subunit 1 (NFKB1), mRNA	F	TCCACAAGGCAGCAGCTAGA	20	247	XM_017347386.1
		R	CCTTCCGGTGGGCAATACAG	20		
8	Cytochrome P450 (CYP1A1) (LOC100342572), mRNA	F	AAAGAGTACACACTCGCAAGA	21	248	XM_002718772.3
		R	AGTTTCTCTTCGATCTCGGGG	21		
9	Glutathione S-transferase pi 1 (GSTP1), mRNA	F	TGTCCCAGAACAAGGATGGC	20	248	XM_002724272.3
		R	AGGTCCCACAAACCCTCACT	20		
10	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA	F	GCTCCCGTTGCTGTCG	16	214	NM_001082253.1
		R	ATACTGGAACATGTAGACCATGTAG	25		

Table 3: The mating behaviours and reproductive performances of the rabbit does supplemented with *Chlorella vulgaris* biomass during gestation.

	Control	T1	T2	T3	T4	Mean	*p*-values
Sexual receptivity (seconds)	86.64±30.20	57.08±26.98	48.57±1.32	44.14±6.28	43.21±14.40	55.93±15.83	0.05
Number of mounting (counts)	1.00±0.16	2.00±0.18	2.00±0.18	2.00±0.12	2.00±0.19	2.00±0.08	0.48
Gestation length	31.28±0.28^{ab}	33.00±1.03^{b}	32.14±0.82^{ab}	32.29±0.71^{ab}	30.42±0.20^{a}	31.79±0.31	0.01
Litter size	6.00±0.97	5.00±1.54	5.00±1.39	5.00±1.40	6.00±0.52	4.40±0.52	0.67
Viability rates	80.95±13.82	63.88±20.38	64.62±16.90	55.10±19.57	100.00±0.00	73.17±7.14	0.28
Kindling rates	85.71±0.26^{b}	83.33±0.34^{b}	57.14±0.16^{c}	59.14±0.26^{c}	100.00±0.00^{a}	76.46±2.98	0.001
Reproductive index	4.53±0.83^{b}	3.75±1.26^{b}	2.61±0.76^{c}	2.04±0.78^{c}	6.14±0.59^{a}	3.82±0.43	0.01
Gestation gain	420.65±20.84	335.93±57.58	300.97±68.21	321.88±37.73	426.91±39.91	362.01±21.83	0.21
Daily feed intake	93.05±3.74	94.99±2.73	91.69±3.80	88.94±1.74	91.88±4.40	92.02±1.48	0.81

^{a,b,c} Means with a different superscript in the same row are different ($p < 0.05$) for the parameters measured. Control - no supplementation; T1 – rabbit does supplemented with 200 mg/kg *Chlorella vulgaris* biomass; T2 - rabbit does supplemented with 300 mg/kg *Chlorella vulgaris* biomass; T3 - rabbit does supplemented with 400 mg/kg *Chlorella vulgaris* biomass; T4 - rabbit does supplemented with 500 mg/kg *Chlorella vulgaris* biomass.

Table 4: Effect of gestational *Chlorella vulgaris* supplementation on serum oxidative stress biomarkers of the rabbits does.

	Control	T1	T2	T3	T4	Mean	p-values
Malondialdehyde (nmol/mL)	23.85±2.61^{a}	13.54±0.32^{b}	14.05±1.59^{b}	11.82±1.68^{b}	13.54±2.19^{b}	15.36±1.23	0.003
Protein carbonyl (µmol/mL)	30.32±4.76^{b}	9.01±11.96^{a}	7.53±14.13^{a}	8.30±21.81^{a}	5.62±14.40^{a}	12.15±13.41	0.06
Total antioxidant capacity (µmol/mL)	11.68±2.81^{b}	102.05±22.47^{a}	58.28±27.72^{ab}	48.42±9.92^{ab}	85.01±14.42^{a}	61.09±10.06	0.02

^{a,b,c} Means with a different superscript in the same row are different ($p < 0.05$) for the parameters measured. Control - no supplementation; T1 – rabbit does supplemented with 200 mg/kg *Chlorella vulgaris* biomass; T2 - rabbit does supplemented with 300 mg/kg *Chlorella vulgaris* biomass; T3 - rabbit does supplemented with 400 mg/kg *Chlorella vulgaris* biomass; T4 - rabbit does supplemented with 500 mg/kg *Chlorella vulgaris* biomass.

Table 5: Effect of maternal gestational *Chlorella vulgaris* intakes on growth performance characteristics and serum estradiol and testosterone of the rabbit kits

Parameters	Control	T1	T2	T3	T4	Mean	*p* - value
Birth weight (g)	34.89±3.40^{b}	40.22±1.81^{a}	39.33±3.44^{a}	41.73±4.37^{a}	50.23±1.67^{a}	41.28±1.69	0.04
Weaning weight per kit (g)	413.55±137.85	419.81±139.93	550.20±100.24	502.26±107.64	566.40±102.4	490.44±37.85	0.62
Weight gain per kit (g)	1169.50±54.22^{b}	1387.74±42.28^{a}	1310.15±18.64^{a}	1386.32±42.87^{a}	1426.26±27.26^{a}	1335.99±37.05	0.003
Daily feed intake (g)	63.85±2.2^{5}b	57.24±2.26^{ab}	45.15±2.2^{2}a	52.21±2.05^{ab}	49.77±2.28^{a}	53.64±2.10	0.03
Feed conversion ratio	2.60±0.1^{0}b	2.00±0.14^{a}	1.66±0.12^{a}	1.76±0.18^{a}	1.63±0.1^{0}a	1.93±0.11	0.01
Oestrogen (pg/mL)	2.30±0.004^{b}	2.32±0.002^{a}	2.32±0.002^{a}	2.32±0.002^{a}	2.32±0.002^{a}	2.32±0.002	0.001
Testosterone (ng/mL)	14.17±0.13^{c}	15.20±0.10^{ab}	14.87±0.12^{b}	15.70±0.10^{a}	15.26±0.14^{ab}	15.04±0.12	0.001

^{a,b,c} Means with a different superscript in the same row are different ($p < 0.05$) for the parameters measured. Control – kits of rabbit not supplemented. T1 – kits of rabbits supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 – kits of rabbits supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 – kits of rabbits supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 – kits of rabbits supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

$p = 0.01$). There was also a significant difference in post-weaning body weight gain of the rabbit kits (1426.26±24.32 vs 1169.50±24.32 g, $p = 0.003$). Furthermore, there was a significant difference observed in the serum concentration of testosterone and oestrogen of the kits kindled by the rabbits supplement does compared with the kits kindled by the rabbit does of the control group (testosterone, 15.70±0.14 vs 14.17±0.14 ng/mL; estrogen, 2.32±0.002 vs 2.30±0.002 pg/mL, $p = 0.001$).

The Serum Biochemical Profile of the Rabbit Does

There was a significant difference observed in the circulating biomarkers of oxidative stress in the serum of the rabbit does (Table 5). There was also a significant difference observed in the relationship between the serum oxidative stress status and reproductive index of the rabbit does. In the control group, as the lipid peroxidation increases, total antioxidant capacity reduced with corresponding lower reproductive index of the rabbit does (Figure 1A).

The Serum Biochemical Profile of the Rabbit Kits

Serum biochemical profiles of the rabbit kits were determined to investigate the health status of the rabbit kits and it was observed that there was no difference in the serum enzymes and some of the parameters determined in the rabbit kits across the groups (Table 6). Although, there was a significant difference observed in the total bilirubin concentration of the rabbit kits ($p < 0.05$), the control group had a higher total bilirubin concentration in the serum compared to treatment (Figure 2E). There was an observation of significant difference in the serum malondialdehyde (MDA) concentration ($p = 0.002$), protein carbonyl content ($p = 0.001$) with corresponding higher activities of superoxide dismutase ($p = 0.04$), and glutathione concentration ($p = 0.001$) of the rabbit kits (Tables 7). Similarly, there was a significant difference observed in the liver malondialdehyde concentration ($p = 0.001$), protein carbonyl content ($p = 0.001$), and antioxidant enzyme activities ($p = 0.004$) of the rabbit kits (Table 8).

The Relative Expression of Selected Functional Genes in the Rabbit Kits

There was significant difference observed in the expression of the selected functional genes in the rabbit kits ($p < 0.05$). The mean expression of the selected functional genes was Gstp1 - 2.00±0.95 folds changes ($p = 0.002$), Cypa1a - 6.15±1.39 folds changes ($p = 0.05$), Nr3c1 - 3.75±1.08 folds changes ($p = 0.001$), AR - 2.55±1.82 folds changes ($p = 0.001$), Ghr - 3.07±0.71 folds changes ($p = 0.03$), Il2 1.80±0.21 folds changes ($p = 0.001$) and Il6 - 2.08±0.32 folds changes ($p = 0.04$). The expression of these genes in the kits of the rabbit does in the treatment groups were compared with the kits of the rabbit does in the control group using value (fold change =1) which are presented in Figure 1B-1F and Figure 2A-2D.

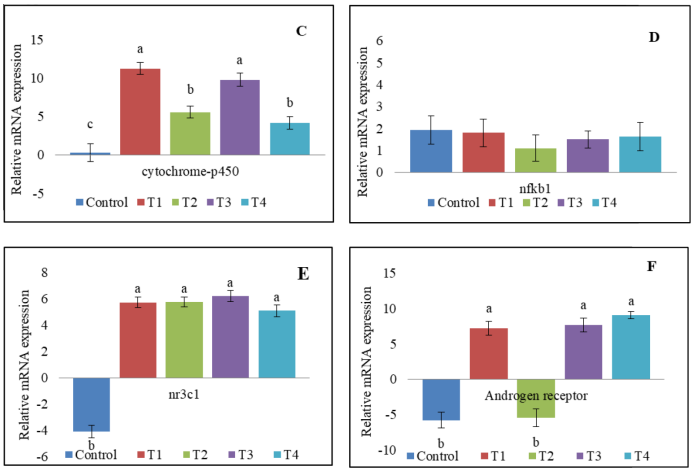

Figure 1: The relationships between the oxidative stress biomarkers and reproductive index of the rabbit does and relative expression of selected functional genes in the rabbit kits.
Bars with different labels are significantly different ($p < 0.05$). (A) This represents the relationship between oxidative stress biomarkers and the reproductive index of the rabbit does; the biomarkers determined are malondialdehyde (MDA), protein carbonyl (PCO), and total antioxidant capacities (TAC). (B) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of glutathione-S-transferase (*GSTP1*) gene in the rabbit kits. (C) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of cytochrome p450 gene in the rabbit kits. (D) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of nuclear factor-kappa beta 1 gene in the rabbit kits. (E) Effects of maternal *Chlorella vulgaris* on the relative expression of the nuclear receptor subfamily 3 group member 1 gene in the rabbit kits. (F) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of the androgen receptor gene in the rabbit kits.

DISCUSSION

This study suggests that maternal gestational intakes of *Chlorella vulgaris* biomass could improve reproduction performances of rabbit does because of the higher reproductive index observed in the supplemented group with the highest intake of the micro-alga *Chlorella vulgaris*. This enhancement is worthy of note, considering the importance of the reproductive index as an account of the totality of success or failure of

Table 6: Effect of maternal gestational *Chlorella vulgaris* supplement intakes on liver oxidative stress biomarkers and antioxidant enzyme activities of the rabbit kits.

Parameters	Control	T1	T2	T3	T4	*p*-value
Malondialdehyde (nmol/mg tissue)	11.26±0.22[b]	9.12±0.10[a]	8.05±0.17[a]	8.68±0.67[a]	8.51±0.28[a]	0.001
Protein carbonyl (nmol/mg protein)	3.51±0.72[b]	0.55±0.22[a]	0.37±0.18[a]	0.38±0.15[a]	0.54±0.14[a]	0.001
Total antioxidant capacity (µmol/mg)	11.50±1.30	14.25±3.56	14.81±1.57	13.42±1.31	12.95±1.00	0.80
Superoxide dismutase (U/mg)	3.39±0.04[c]	3.89±0.02[b]	4.59±0.25[ab]	4.57±0.30[ab]	5.07±0.32[a]	0.004
Catalase (U/mg)	2.18±0.44	1.93±0.08	2.77±0.32	2.19±0.18	2.71±0.43	0.35
Reduced glutathione (µmol/g)	10.59±0.15	11.38±0.24	10.81±0.38	10.56±0.16	11.33±0.18	0.10

[a,b,c] Means with a different superscript in the same row are different ($p < 0.05$) for the parameters measured. Control – kits of rabbit not supplemented. T1 – kits of rabbits supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 – kits of rabbits supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 – kits of rabbits supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 – kits of rabbits supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

Table 7: Effect of maternal gestational *Chlorella vulgaris* supplement intakes on serum oxidative stress biomarkers and antioxidant enzyme activities of the rabbit kits.

Parameters	Control	T1	T2	T3	T4	*p*-value
Malondialdehyde (nmol/mL)	26.69±4.02[c]	22.01±1.15[b]	18.55±4.71[b]	19.51±2.24[b]	5.00±0.65a	0.002
Protein carbonyl content (nmol/mL)	77.38±5.52[b]	58.75±2.22[a]	69.88±4.89[a]	76.02±2.27[b]	62.27±2.92a	0.001
Total antioxidant capacity (µmol/mL)	72.92±16.07	75.08±2.05	72.41±2.92	85.27±9.20	101.31±5.15	0.14
Superoxide dismutase (U/mL)	13.29±0.95[ab]	17.83±2.29[b]	13.06±1.47[ab]	13.21±1.31[ab]	17.06±0.38[b]	0.04
Catalase (U/mL)	4.30±0.59	4.70±0.60	4.78±0.53	3.21±0.58	4.54±0.16	0.28
Reduced glutathione (µmol/mL)	6.22±0.61[c]	11.63±0.81[b]	13.73±0.24[a]	14.50±0.51[a]	10.13±0.17[b]	0.001

[a,b,c] Means with a different superscript in the same row are different ($p < 0.05$) for the parameters measured. Control – kits of rabbit not supplemented. T1 – kits of rabbits supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 – kits of rabbits supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 – kits of rabbits supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 – kits of rabbits supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

Table 8: Effect of maternal gestational *Chlorella vulgaris* supplement intakes on blood biochemical profiles of the rabbit kits.

Parameters	Control	T1	T2	T3	T4	SEM	*p* - value
ALT (IU/L)	29.14	24.07	25.06	28.28	26.23	0.92	0.40
AST (IU/L)	12.91	14.82	18.14	14.60	15.48	0.97	0.61
ALP (IU/L)	58.67	54.40	61.07	45.87	56.27	2.25	0.26
Serum Urea (mg/dl)	37.06	35.11	33.83	35.54	33.04	0.81	0.62
BUN (mg/dl)	17.31	16.40	15.80	16.60	15.43	0.37	0.63

[a,b,c] Means with a different superscript in the same row are different ($p < 0.05$) for the parameters measured. Control – kits of rabbit not supplemented. T1 – kits of rabbits supplemented with 200 mg/kg BW *Chlorella vulgaris* biomass. T2 – kits of rabbits supplemented with 300 mg/kg BW *Chlorella vulgaris* biomass. T3 – kits of rabbits supplemented with 400 mg/kg BW *Chlorella vulgaris* biomass. T4 – kits of rabbits supplemented with 500 mg/kg BW *Chlorella vulgaris* biomass.

reproduction performances.[21] In rabbits, the reproductive index as a product of kindling, kits viability, and litter size of a given rabbit doe could be accounting for both quantitative and qualitative reproduction performances.[12] Antioxidants in the alga could be responsible for the improved reproduction performances and these agreed with previous reports exploring effect of supplemental antioxidant-rich micro-alga intake on the reproduction performance of rats which concluded that the gestational intake of the antioxidant-rich micro-alga could lead to improved gestation outcome performances.[22]

The free radical scavenging power of the micro-alga could be attributed to improved reproductive outcomes because of the reduction in the accumulated malondialdehyde observed in the supplemented group which was also similar to reports of of some researchers which described malondialdehyde as a compound of reproduction risk because of its negative effect on gestation.[23] Therefore, the reduction of this compound in the supplemented group of this study suggests that *Chlorella vulgaris* intakes due to its antioxidant properties could serve as a health promoter during the gestation period. Similar finding was also reported in an earlier study which suggested that reduced lipid peroxidation in pregnant rats could be associated with better gestational outcomes.[24] Similar research on elucidation of antioxidant intakes in pregnant rat models, reported to eliminate birth defects due to reduced malondialdehyde.[25,26]

The maternal gestational intake of *Chlorella vulgaris* biomass in this study also led to the production of rabbit kits with higher birth weight despite a non-significant difference in the litter sizes of the rabbit does. Apart from the recorded higher birth weight, the rabbit kits of the supplemented group also attained higher body weight gain in the growth phase with reduced oxidative stress status. This demonstrated that a systemic improvement of birth weight and growth rates of rabbits and other mammals is possible through supplemental intake of antioxidant-rich micro-alga. The antioxidant principles could have acted on reducing oxidative stress damage on the fetus right from the implantation till the end of the gestation period which could have programmed the rabbit kits for better growth.[27]

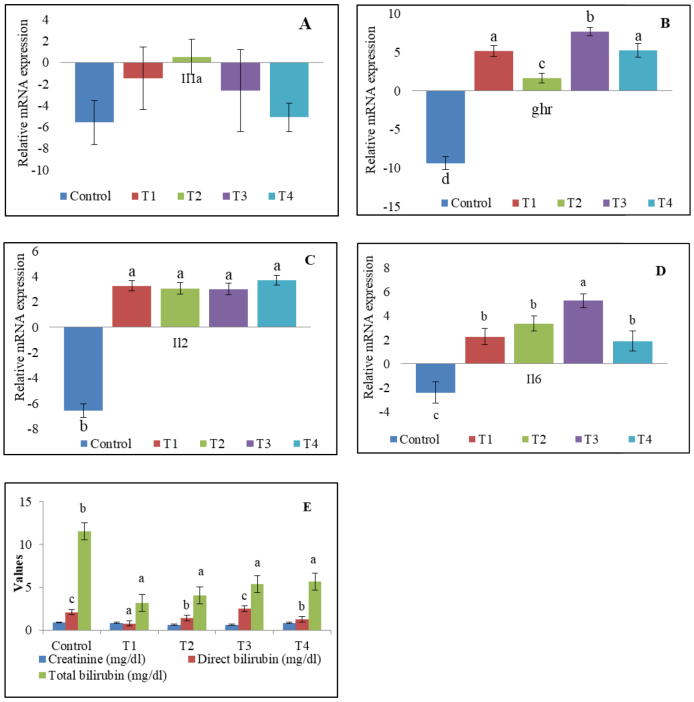

Figure 2: The relative expression of selected target genes and blood biochemical profile of the rabbit kits.
Bars with different labels are significantly different ($p < 0.05$). (A) Effects of maternal *Chlorella vulgaris* supplements on relative expression interleukin-1-A gene in the rabbit kits. (B) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of growth hormone receptor gene in the rabbit kits. (C) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of interleukin-2 gene in the rabbit kits. (D) Effects of maternal *Chlorella vulgaris* supplements on the relative expression of interleukin-6 gene in the rabbit kits. (E) Effects of maternal *Chlorella vulgaris* supplements on serum creatinine and bilirubin levels of the rabbit kits.

For the rabbit kits, the linkage between *Chlorella vulgaris* and oxidative stress protection could be traced to the antioxidant protection of the kits against free radicals *in-utero*; because free radicals generated at the placental-maternal interface are inherent biochemical activity for the protection of developing fetuses – a situation which necessitate their eliminations. However, if otherwise, these free radicals could be involved in mechanisms limiting the flow of blood and nutrients to the developing fetus, thereby reducing fetal growth and induction of low birth weight and adulthood systemic malfunctions. *Chlorella vulgaris* intake in this study could be associated with the production of kits with higher birth weight and reduced oxidative stress status *in-utero*, because this could increase the flow of blood and nutrients to the developing fetus which could subsequently lead to enhanced offspring birth weight, pubertal advancement, and immune functions in humans and animals.[32,33]

Beyond physical and biochemical evaluations, molecular investigations in this study also revealed that there was up-regulation of selected functional genes involved in the complex processes of antioxidant protection, growth regulation, and immune regulation in the kits of the supplemented rabbit does – which are indications that the micro-alga induced molecular protection against oxidative stress. The up-regulation of *Gstp1* in the kits of the supplemented group could be linked with possible *in-utero* antioxidant protection imprint conveyed on to the rabbit kits due to the *Chlorella vulgaris* intake by the dams. This protection could be linked with the effect of placenta inhibition of oxidative stress damage on the rabbit kits; which can be considered as a fetal programming attempt using the micro-alga. The interaction of the gene *Gstp1* with other genes including *Cyp1a1*, *Cyp1b1*, *Cyp2e1*, *Ephx1*, *Gstk1*, *Gsto1*, and *Gsto2* showed that the gene could initiate actions against oxidative stress, inflammation, and detoxification of xenobiotic agents.[38,39]

CONCLUSION

The supplementation of the *Chlorella vulgaris* biomass improved reproduction and growth performances of rabbits. The maternal gestational intake of *Chlorella vulgaris* also leads to the production of rabbit kits with higher birth weight and growth rates promoted by reduced malondialdehyde as a biomarker of oxidative stress. The improvement of growth and oxidative stress protection was imprinted in the rabbit kits at the molecular, cellular, and systemic levels as confirmed by the up-regulation of some genes associated with stress and growth regulations in the rabbit kits of the supplemented groups.

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Ethics and Animal Use Protocol Approval

Ethical approval for the animal study was obtained from a joint committee sitting of the Institutional Animal Ethics Committee (IAEC) of the ICAR – National Institute of Animal Nutrition and Physiology, Bangalore, India, and Committee for Control and Supervision of Experiments on Animals (CPCSEA) - an agency of the Central Ministry of Environment, Forests and Climate Change; Government of India. The registration number of the ethics approval given by the committee is 1437/GO/Re/SL/11/CPCSEA.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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