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Research Article

Study of *Drosophila melanogaster* response to electric stress and efficacy of Abhrak bhasma and ascorbic acid as dietary supplement

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ABSTRACT

With an increase in usage of variety of electric appliances, not only humans but also other coexisting organisms too face an occasional exposure to electric shock. Majority of stresses manifest directly or indirectly through oxidative stress. This study largely deals with understanding the effect of electric stress on catalytic as well as non-catalytic components of antioxidant machinery in insect using *Drosophila* as a model system. The effect of dietary supplement of ascorbic acid and abhrak bhasma in management of elwectric shock has also been evaluated. The activity of superoxide dismutase (SOD) and catalase enzymes were assessed as a part of catalytic component while, total GSH content, GSH:GSSG ratio, free radical scavenging capacity were studied as a part of non-catalytic component of antioxidant machinery of cells. Along with these parameters, the molecular effect at transcription levels of cncC (Nrf-2), hsp70 genes as well as the effect on lipid peroxidation was evaluated in response to electric shock. It was observed that ascorbic acid as a dietary supplement, in general, show protective role in electric stress. Abhrak bhasma, on the contrary, negatively affect various antioxidant parameters. Administration of these compounds and subsequent electric shock can thus be tried for demographic manipulations of insects.

Keywords: *Drosophila;* oxidative stress; abhrak bhasma; electric stress.

INTRODUCTION

In the developing world there has been a tremendous increase in manufacture as well as usage of electricity based instruments and appliances. Consequently, exposure to electric field by humans and other organisms has become more frequent. These organisms, coming in contact with electric field, are prone to various stresses under electric field (Elwood and Adams 2015; Kim *et al.* 2017; Tsong and Su 1999). Exposed to a very strong electric field, an organism suffers from a failure of various organs or destruction of vital tissues and may die. However, moderate exposure to electric field may only trigger various changes in the physiology of an organism (Petri *et al.* 2017).

An organism exposed to any type of stress exhibits alterations in various oxidative parameters. These changes may have important implications in various aspect of life expectancy of an organism (Harman 1956). *Drosophila melanogaster* flies have been used as a model in research in biological disciplines including genetics, physiology and aging. Researchers have worked on electric shock as a negative enforcement to link it with olfactory learning in *Drosophila* model system (Pauls *et al.* 2010; Malik and Hodge 2014). The electric shock has also been shown to induce behavioral aftereffects in motor function in *Drosophila* flies (Chadha and Cook 2014). There is, however, a general paucity of reports involving evaluation of the effects of electric shock on oxidative parameters of *Drosophila*.

Various indices and parameters can be analyzed in order to comprehend the effect of oxidative stress. These parameters can be broadly classified into catalytic and non-catalytic categories. Catalytic group mostly includes the enzyme systems like SOD, Catalase, and Peroxidases that specifically target a particular type of reactive oxygen species and Glutathione reductase that regenerates the reduced glutathione molecules involved in non-catalytic antioxidant activity. The non-catalytic mechanism of managing oxidative stress, on the other hand, includes Glutathione system, thioredoxin system, certain vitamins and other molecules that target free radicals in a nonspecific manner.

Another parameter that can reflect oxidative stress is the levels of lipid peroxidation that targets the membrane systems in cells. With age, the lipid peroxidation increases due to accumulation of free radicals as the organism loses the capacity to deal with the free radicals that are being continuously generated (Spiteller 2001; Kale and Rajendran 2002). Any external stress can expedite these processes and have deleterious

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effect on the lifespan of an organism. Understanding the catalytic and non-catalytic parameters along with the extent of lipid peroxidation can help in comprehending the ability of an organism to fight with stresses.

Supplementation of diet with natural or artificial antioxidants may restore the antioxidant potential of the cells and may increase the life expectancy of cells as well as of the organism (Nijsure and Kale 2005). Certain naturally occurring compounds like vitamins have been shown to boost the antioxidant machinery (Kartha and Krishnamurthy 1977; Liebler 1993; Padayatty *et al.* 2003). Ascorbic acid (ASC), a known natural antioxidant, has been most popularly used for mitigating various stress related parameters due to its inherent reducing capacity (Tsai *et al.* 2011).

The present investigation is an effort to comprehend changes in various oxidative parameters at physiological and molecular levels, in response to electric stress using *Drosophila* as a model system. The study also evaluates the efficacy of dietary supplement of abhrak bhasma (AB), an Ayurvedic preparation, during electric stress. Subsequently, the results obtained for abhrak bhasma supplementation has been compared to dietary supplement of ASC.

MATERIALS AND METHODS

Abhrak bhasma

Sahastraputi Abhrak bhasma was procured from Dhootapapeshwar Ltd, among the leading manufacturers of ayurvedic medicines (Batch no: P150300110).

Drosophila husbandry

Drosophila melanogaster (Canton S strain) flies were maintained on corn-meal agar medium at 26 ^{0}C with 12 hour light and 12 hour dark cycle. The control flies were allowed to lay eggs on a medium containing 0% AB, 0.1% AB, 0.5% AB and 20 mM ASC for 12 hours. After 12 hours of egg laying, the adults were removed and the eggs were allowed to develop till they attained the age of 2 days of adult life. These 2 day old flies were then subjected to electric shock.

Electric stress

Construction of cage for Electric stress (EShock2):

A cage for subjecting *Drosophila* flies to electric shock was self-designed in the laboratory (Figure 1). This was named Eshock2 and had equally spaced (1mm apart) copper wires sewed to a non-conducting thick paperboard. The paper board was eventually fitted in a polyvinyl box (6.5cm x11.5cm x 3.5cm) with a lid having a hatched aperture for transferring the flies. Ends of the alternate wires were pooled and then drawn out through the gap between box and the lid. These were then connected to the positive and negative terminals of power supply that had provision for applying varying voltage from 0-200 volts.

Stress induction

In four replicates, 2 day old adult flies (150-160) from media containing 0% AB, 0.1% AB, 0.5% AB and 20 mM ASC were removed and segregated into control and test groups. Control groups were transferred to EShock2 box without any electric supply while the test groups were introduced in to EShock2 box for electric stress. The box was connected to power supply set at 110V for 1hour (Figure 2). The EShock2 box was tapped after every 5minutes to ensure that each fly gets a shock at least once in every 5 minutes. Similar procedure was also followed for the flies from control groups. After 1 hour the flies were removed from respective EShock2 boxes and separated (10 each) into vials containing Trizolin for RNA extraction, Protein extraction buffer for Catalase and SOD enzyme assays (20 each), GSH assay extraction buffer for glutathione estimation (20 each) and 0.154 M KCl for DPPH and Lipid peroxidation assays (25 each). The results of the experiments were represented as percent change with respect to flies without electric stress for each feeding regime. The statistical comparisons were then made between responses under each feeding regime.

Catalase assay

Catalase assay was performed by monitoring molecular breakdown of H_2O_2 by catalase at 240 nm, under pH 7, as per the method described by Aebi (1983). The adults (10male, 10 female) were homogenized in protein extraction buffer containing 20 mM tris-acetate buffer (pH7.8), 0.1% triton X-100 and 1mM PMSF using micropestle homogenizer (Sigma catalogue no: Z359955-1EA). Protein values were measured using Bradford's assay. One unit of catalase activity is defined as enzyme required for decomposing 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25 °C and expressed as enzyme units.

SOD assay

SOD assay was performed by measuring auto-oxidation inhibition of pyrogallol as described by Marklund and Marklund (1974) with some modifications. The method involves blocking of auto oxidation of pyrogallol by SOD. The inhibition of oxidation of pyrogallol was followed at 420nm on a Biotek spectrophotometer (Model no: EPOCH –Gen5). One unit of SOD activity was defined as amount of enzyme that reduces the pyrogallol auto-oxidation by 50% at pH 8.5 at 25 °C and expressed as enzyme units.

Glutathione content (Total GSH and GSH:GSSG ratio)

The amount of GSH and its oxidized form GSSG were measured using enzymatic recycling method described by Rahman *et al.* (2006). The adults (10male, 10female) were homogenized in glutathione extraction buffer containing 0.1M phosphate buffer with 5 mM EDTA (pH 7.5), 6 mg/ml sulphosalicyclic acid and 0.1% triton X-100. Oxidation of GSH was carried out with DTNB (5, 5'-dithio-bis-(2-nitrobenzoic acid)) to form a yellow

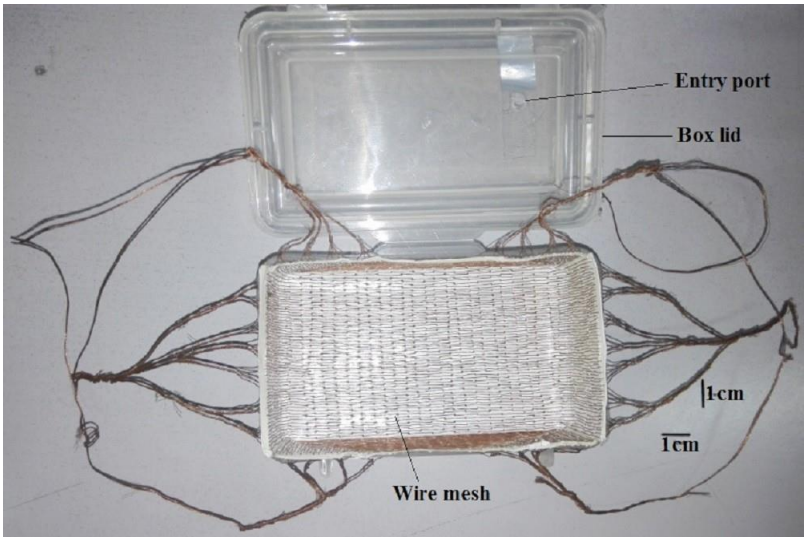

Figure 1: Eshock2 apparatus

The wire mesh crafted on a paper board is fitted inside the polyvinyl box. The two terminals of the wire mesh is fitted to +ve and –ve wire terminals that fits into the power source. Flies can be inserted through entry port made on box lid.

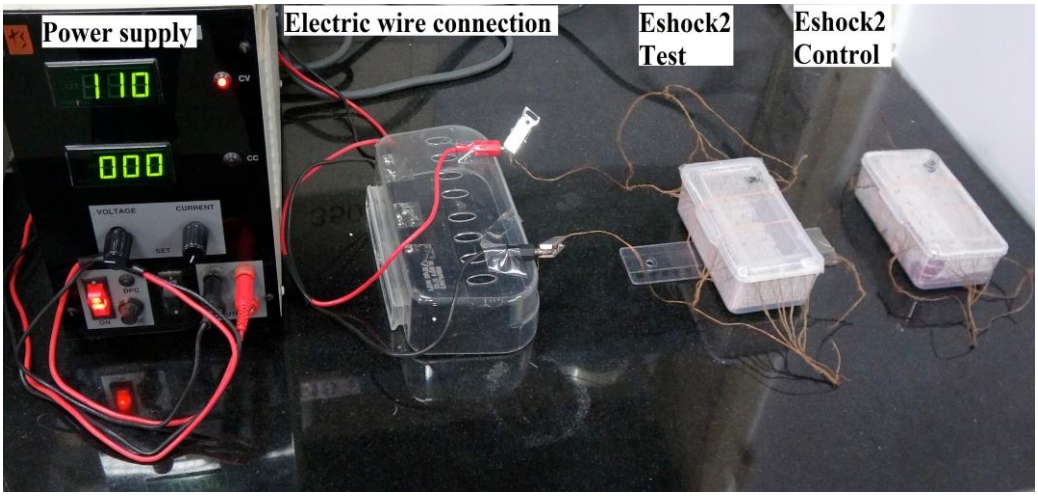

Figure 2: Eshock2 assembly

All alternating small wires are pooled together at opposite sides and connected to the respective poles connected to the power supply unit for the Test set. The control box is same as Test box except that wires are not connected to power supply

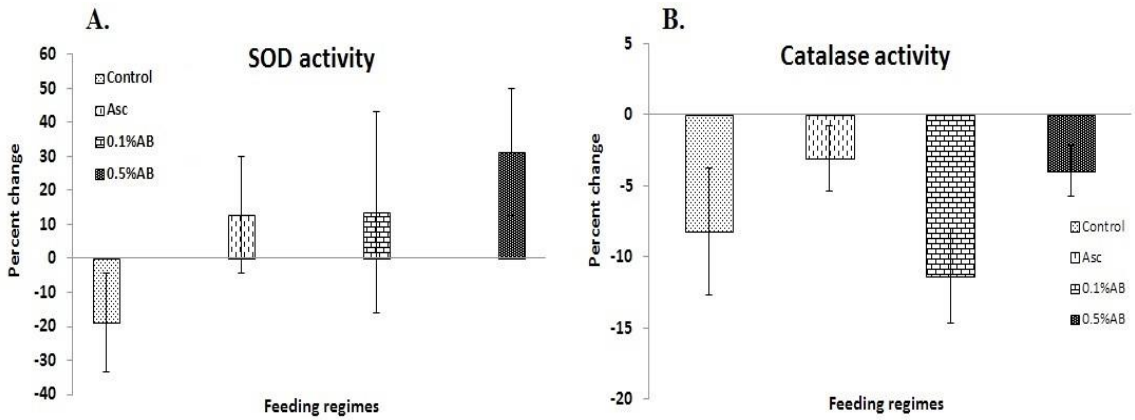

Figure 3: Percent change in Catalytic components. A. SOD activity B. Catalase activity

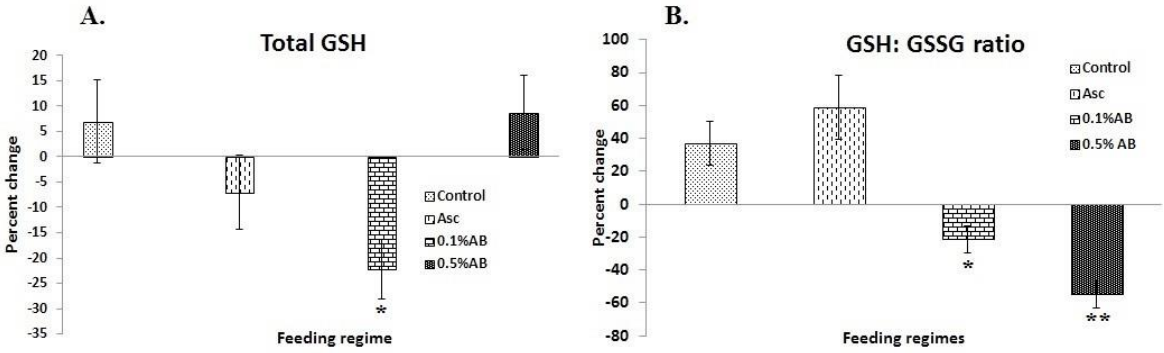

Figure 4: Percent change in non-catalytic components A. Total GSH; B. GSH:GSSG ratio

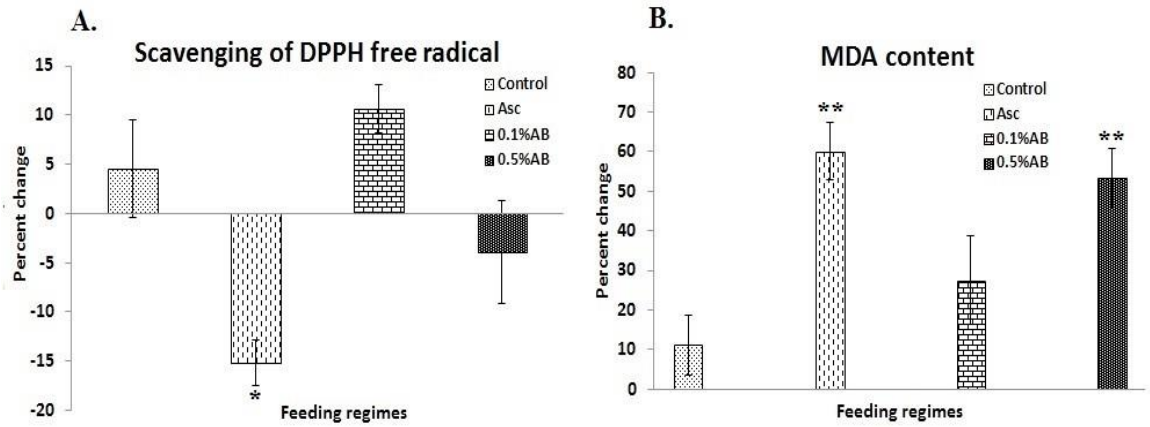

Figure 5: Percent change in oxidative stress indicators

A. DPPH scavenging- analysed as mg of protein (equivalent to amount of tissue used) required for scavenging DPPH by 50%. Increase in the amount of protein required represents lower DPPH scavenging capacity. **B. Percent change in the extent of lipid peroxidation (**The extent of lipid peroxidation is measured as amount of MDA content in tissue). (* Tukey hsd inference: p<0.05, **Tukey hsd inference: p<0.01)

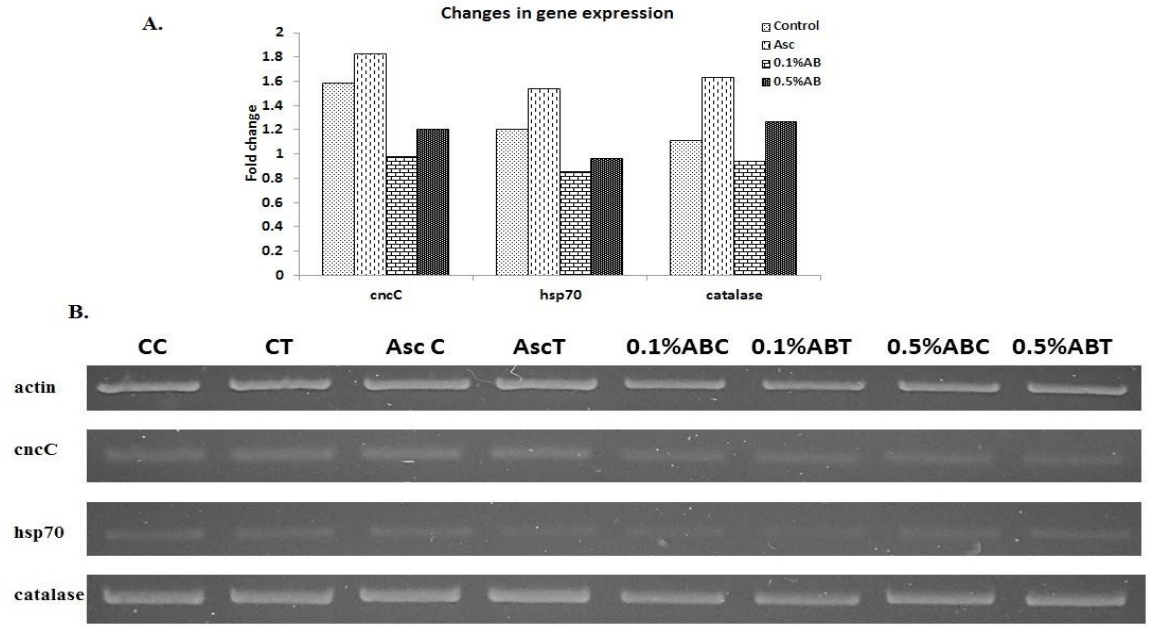

Figure 6: Changes in expression of stress induced genes

A. Real time PCR results: measured in terms of fold change in the expression levels of genes with respect to stress control in each feeding regime **B. semiquantitative RT-PCR results:** the cDNA synthesis was followed by PCR amplification of each gene, electrophoresis and analysis using ImageJ software

Table 1: Genes and primers

Gene	Product size	Forward	Reverse
Actin	469bp	CGGCTCGGACAGTGATAGAC	CCGGTACCAAGTATCCTCGC
cncC	147bp	AGCGCTAGGCTAAAGCAACA	GACAGTTAACGGGACGCTCT
hsp 70	188bp	TTGACAACCGGCTAGTCACT	GGTGTAGAAGTCTTGGCCCT
Catalase	366bp	GAACTGCCCGTACAAGGTGA	GTCAGCATGCGACCGAAATC

Table 2: Percent changes in the oxidative parameters due to electric shock

Parameters Studied (percent change)	Control	ASC	0.1%AB	0.5%AB
SOD activity	-18.9±14.4	12.8±17.0	13.5±29.5	31.4±18.6
Catalase activity	-8.2±4.4	-3.1±2.3	-11.3±3.3	-4±1.8
DPPH scavenging activity	4.5±5	-15.2±2.3	10.6±2.4	-4.0±5.2
MDA content	11.2±7.6	60.1±7.1	27.5±11.2	53.4±7.4
Total GSH	7.0±8.3	-7.0±7.3	-22.3±5.9	8.7±7.3
GSH:GSSG ratio	36.8±13.2	58.8±19.2	-21.3±8.1	-54.8±8.2

Numbers after '±' indicate standard error values

derivative, TNB (5-thio-2-nitro-benzoic acid), measurable at 412 nm. The GSSG, thus formed, is recycled using Glutathione reductase enzyme in presence of NADPH. The rate of formation of TNB in comparison to standards was correlated to find the concentration of the GSH and GSSG in samples. The free GSH was derivatized with 2-vinylpyridine for accurate measurement of GSSG. The excess of 2-vinylpyridine was later neutralized with triethanolamine.

DPPH scavenging assay

Free radical scavenging capacity of the tissue was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (Brand-williams *et al.* 1995). The adults (15male, 15female) were homogenized in 0.154M KCl using micropestle homogenizer and the whole body extract was used for the assay. The varying volumes (20 µl, 40 µl and 100 µl) of tissue extract were used for DPPH scavenging. The amount of tissue required (in terms of mg of protein) for scavenging 50% DPPH was measured at 517 nm on BioTek Spectrophotometer.

Lipid peroxidation

Lipid peroxidation was measured by TBARS (Thiobarbeturic acid reactive substances) assay method (Zeb and Ullah 2016; Devasagayam *et al.* 2003). The whole body extracts of adults (15 males, 15 females) homogenized in KCl and was made to react with TBA reagent (containing 0.037 g TBA, 15% TCA and 0.24 N HCl per 10ml). Reaction mixture of samples as well as different concentrations of standard MDA were boiled for 15minutes and the MDA was measured at 532 nm on BioTek spectrophotometer. Concentration of MDA in the samples was estimated using the standard MDA plot.

RT PCR and qRT PCR

The total cellular RNA was extracted using TRIZOL method as per manufacturer's instruction (Merck) subsequently the cDNA was prepared using SD-prodigy cDNA synthesis kit (SDFine Chem Ltd.). Primers for actin, cncC, hsp 70 and catalase (Table 1) were designed using NCBI primer design tool.

Amplification of these genes was carried out with the help of PCR using the reaction setting as 94°C for 2 minute, repeated cycle of (25 cycles): each of 94°C for 30 second, 62°C for 30 second and 72°C for 30 second with final extension at 72°C for 5 minute. The products thus obtained were then run on 2% agarose gel. The gel was observed under UV trans-illuminator and the images were captured in Uvitech system. The semi-quantitative estimation of changes in gene expression was carried out in the captured gel images using 'ImageJ' software. Following this, qRT PCR was carried out using syber based EVAGREEN (Biorad) on CFX96 Real Time System (Biorad). The cT values were analyzed for fold change in expression of genes using actin as an internal control. Data from both the experiments were compiled and any mismatch data were repeated for qRT PCR to obtain accurate results. (Triplicates of real-time PCR were not performed due to paucity of funds.)

Statistical analysis

All the experiment and analysis were performed in minimum of triplicates. Statistical significance between two mean values was analyzed using student's t test. For comparing more than two means, ANOVA (Analysis Of Variance) test was performed. ANOVA was followed by Bonferroni and Holm multiple comparison post hoc analysis. For comparing other intergroup differences (between test groups) Tukey HSD post hoc method was performed. Throughout the manuscript, * and ** represents Holm inferences $p<0.05$ and $p<0.01$ respectively; while # and ## represents Tukey HSD inference $p<0.05$ and $p<0.01$ respectively.

RESULTS AND DISSCUSSION

Feeding AB does not alter enzyme activity

The figure 3A shows the result of SOD activity in flies from different feeding regimes, subjected to electric

shock (110v for 1 hour), as percentage of control. In flies from 0% AB feeding regime subjected to electric shock (negative control) showed an apparent drop in the activity of SOD as compared to control (not subjected to electric stress) flies, though it was not statistically significant. Similarly, in the flies from other feeding regimes and subjected to electric shock, there was a slight increase in the activity of SOD in comparison to control flies, though this too was statistically insignificant (ANOVA p-value:0.449603)

As seen in figure 3B and table 2, in comparison to the control flies not receiving any electric shock, the flies from negative control as well as all feeding regimes, showed an apparent decrease in the activity of catalase enzyme. However, this was found to be statistically insignificant as compared to the response of control flies under electric shock (**ANOVA p-value: 0.263906**).

General Free radical scavenging capacity

When the GSH content of the flies in control group was compared to that in control flies subjected to electric stress, an apparent increase was noticed though the difference was not statistically significant (Figure 4A, Table 2). The feeding of AB decreased the total GSH content of flies in response to electric stress (**ANOVA p-value: 0.013420**). The percent change in total GSH content due to electric shock was not significant between flies from control, ASC and AB at 0.5% concentration feeding regimes. However in the flies from 0.1% AB feeding regime a significantly decreased total GSH content was noticed. The percent change in GSH content of 0.1%AB under electric stress differed significantly in comparison to flies from control as well as 0.5% AB feeding regime (Tukey HSD inference: $p<0.05$ and $p<0.05$ respectively).

The GSH:GSSG ratio indicates how efficiently the oxidised glutathione is reduced and made available for reducing the free radicals and/or ROS. The flies from control and ASC feeding regime exhibited an increase in gsh:gssg ratio in response to electric stress (Figure 4B, Table 2). On the contrary, in flies of AB feeding regime under electric shock, there was a decrease in the GSH to GSSG ratio. The percent change in the gsh:gssg ratio obtained in the flies fed with AB and subjected to electric stress was significantly different in comparison to the response of flies from control as well as ASC feeding regimes, under similar conditions (Overall ANOVA p-value: $9.36*10^{-6}$, Tukey HSD inference: $p<0.01$). Although flies from 0.5%AB regime show an apparent decrease by 34% in GSH:GSSG ratio as compared to flies from 0.1%AB feeding regime. The difference, however, was found to be statistically insignificant.

The amount of tissue (measured in terms of amount of protein used in the reaction) required to scavenge 50% of Dpph free radicals indicates the ability of an organism to handle the free radicals generated in the body. The greater amount of tissue required to scavenge 50% of the dpph radicals represents a proportionate decrease in the overall free radical scavenging capacity. The feeding of ASC caused an increment in free radical scavenging capacity as compared to flies from control as well as AB feeding regime under electric shock (overall ANOVA p-value: 0.000934, Tukey HSD inference: $p<0.05$). This percent change in scavenging capacity was also significantly different from response of 0.1%AB flies (Tukey HSD inference: $p<0.01$). However, the flies from AB feeding regimes at 0.1% as well as 0.5% concentration did not show any statistical difference in DPPH scavenging response in comparison to control flies (Figure 5A, Table 2).

Increase in lipid peroxidation under electric stress:

The extent of lipid peroxidation in flies from all feeding regimes was found to increase due to electric stress (Figure 5B, Table 2). The feeding of ASC as well as AB had significant effect on the extent of lipid peroxidation under electric stress (ANOVA p-value: 0.000667). The flies from ASC and 0.5% AB feeding regime showed drastic increase in the MDA levels due to electric stress. The percent change in MDA levels of flies from ASC and 0.5% AB feeding regimes, in response to electric stress, was significantly different in comparison to the control flies under electric shock (Tukey HSD inference: $p<0.01$ and $p<0.01$ respectively). The percent change in MDA contents of flies from 0.1% AB feeding regime under electric shock was statistically insignifcant as compared to control flies.

Molecular changes due to electric stress

Electric shock induced an increase in the levels of cncC transcription in flies from control as well as ASC feeding regimes by more than 1.5 fold when compared with the negative control (Figure 6). The flies from 0.5% AB feeding regime showed about 1.2 fold increase in the cncC gene expression, whereas, the flies from 0.1% AB feeding regime exhibitd no change in the level of expression of cncC under electric stress.

Electric stress also affected in the expression level of HSP70 and catalase genes. The flies from control feeding regime (subjected to electric shock) had 1.2 and 1.1 fold increase in the hsp70 and catalase gene respectively as compared to control flies not subjected to electric stress. The flies from ASC feeding regime showed more than 1.5 fold increase in the transcription level of hsp70 and catalase genes.

The flies from AB feeding regime at both the concentrations, however, had more or less similar expression levels of hsp70 and catalase genes, under electric stress, in comparison to their stress controls. Only the flies from 0.5% AB feeding regime exhibited 1.2 fold increase in expression level of catalase due to electric shock.

Every living cell poses a unique electric charge on them, generated mainly by differences in charged ions across the cell membrane. The difference in the elec-

tric potential on the outer and inner surfaces of a cell membrane (membrane potential) differs according to the cell type and plays a critical role in various functions related to the cell. Among these, muscles (cardiac as well as skeletal) and neuronal cells rely heavily on the membrane potential for their function. Continuous exposure to electric shock mainly affects neurological, cardiac functions and can also lead to burns due to resistance of the body (Pauls *et al.* 2010; Chadha and Cook 2014; Batsching *et al.* 2016). Excessive damage to these functions ultimately leads to death of an organism.

Any stress, let it be chemical, physical or physiological, directly or indirectly affects the parameters of oxidative stress. These parameters have a profound effect on overall health of an organism. Exposure to electromagnetic field has been shown to induce generation of free radicals in the organism and subsequently their effects (Repacholi and Greenebaum 1999; Jajte *et al.* 2002; Harakawa *et al.* 2005). In *Drosophila*, exposure to static electric field has also been linked to changes in biogenic amine levels in the brain (Newland *et al.* 2015).

The current study suggests that electric shock does not affect the activity of SOD enzyme in *Drosophila*. The diet supplemented with AB as well as ASC also does not influence the SOD activity. This might be due to the fact that ROS and/or free radicals are being reduced by certain molecules like GSH. The activity of catalase enzyme has been noticed to decrease in the flies from all the feeding regimes tested under electric stress. This is indicative of either increased lipid peroxidation or an alternative mechanism (like peroxidase activity) of disposal of H_2O_2.

At the transcriptional level, the flies from ASC feeding regime are seen to exhibit an increase in the catalase gene expression in response to electric stress. The flies from AB feeding regime, subjected to electric stress, however do not have any significant change in the level of transcription of catalase gene.

The non-catalytic components of an organism are among the first chemical barriers that protect an organism from the damaging effect of free radicals, generated in response to various stresses. Electric shock does not cause major changes in the total GSH content, suggesting that the breakdown and regeneration of GSH is matched under such conditions. In the flies from diet supplement of AB at 0.1% AB, subjected to electric stress, a significant decrease in the total GSH content, as compared to flies from control as well as the flies in 0.5% AB feeding regime. The GSH:GSSG ratio is a better indicator of stress response than the total GSH content (Owen and Butterfield 2010). The flies from control as well as ASC feeding regime indicated higher GSH:GSSG ratio in response to electric shock, indicative of efficient regeneration of GSH from GSSG. On the contrary, flies from AB feeding regime, subjected to electric stress, exhibited lower GSH:GSSG ratio indicating a less efficient reduction of GSSG to GSH. This suggests that the electric shock exerts a more severe stress in the flies from AB feeding regime in comparison to the flies from other feeding regimes.

The general free radical scavenging capacity of organism is also affected in response to electric shock. Under electric stress, the changes in the free radical scavenging capacity differ with respect to the dietary supplements. AB does not seem to cause any significant change in the free radical scavenging capacity as compared to control flies. However, electric shock seems to trigger an increase in the free radical scavenging capacity in flies from ASC feeding regime in comparison with the flies from control and 0.1% AB feeding regimes.

The damages caused by ROS/free radicals also culminate into increase in lipid peroxidation resulting into deleterious effects on the cells and tissues. The electric stress in general, increases the lipid peroxidation in *Drosophila*. The flies from AB feeding regime and ASC feeding regime that are exposed to electric shock show significant increase in lipid peroxidation compared to the flies from control feeding regime. It is suggestive of the fact that electric stress cannot be effectively handled by the tissues even after supplementing the diet with antioxidants chosen in the study.

Interestingly, even after positively influencing the other oxidative parameters, the standard antioxidant, ASC, has failed to prevent the extent of lipid peroxidation in response to electric stress. This inefficiency remains the matter of further investigation. AB, on the other hand, seems to enhance the deleterious effects of electric shock on all vital parameters of *Drosophila*. This effect is also apparent immediately after the flies are exposed to electric field. The flies from 0.5% AB feeding regime, especially, exhibit noticeable avoidance response and in increasing numbers they seem to be paralyzed due to the electric shock.

Similar responses have also been observed at the molecular level, in the transcription of cncC (Nrf-2) and hsp70, which are the stress regulating genes. The transcription of these genes, in general, increases under various stress conditions. In the present study, both these genes seem to have been upregulated in the flies from control and ASC feeding regimes in response to electric shock thus better equipping the cellular defense. AB on the other hand, does not bring about any major upregulation of these genes and thus proves to be less or not protective against electric stress.

The analysis of composition of AB indicates the higher metal contents, especially Iron, in various forms (Bhatia and Kale 2013). As the metals are very good conductors of electricity, the consumption of the AB might have resulted in dramatic effects of electric shock.

CONCLUSION

In general, it is evident that electric shock causes wide

array of changes, at physiological as well as molecular levels, linked to oxidative stress in the organism. The feeding of AB has negative impact on these oxidative parameters under electric stress in contrast to the other stresses (Bhatia *et al.* 2013). This is contrary to the improvement in behavioral and physiological response of *Drosophila* due to feeding of AB in absence of any stress (Subedi *et al.* 2017). The feeding of natural antioxidant compound, like ASC, has a significant protective effect in order to cope with electric stress. However, ASC feeding has also been found to cause higher lipid peroxidation under electric shock. If the insects are being exposed to electric stress, feeding abhrak bhasma would enhance its effect while feeding ascorbic acid may extend some protection. More detailed investigations, however, is required with respect to other molecular changes in order to use these compounds as hypothesized here.

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