SELENIUM ATTENUATED 2, 4-DICHLOROPHENOXYACETIC ACID INDUCED TOXICITY IN EXPERIMENTAL RAT MODEL: ROLE OF Na^+/K^+-ATPase ACTIVITY AND ELECTROLYTES STABILITY

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ABSTRACT

2,4-Dichlorophenoxyacetic acid (2,4-D), a synthetic auxin herbicide is commonly used in agricultural lands due to its economic value, good selectivity and a wide range of weed control. Its continual usage induces various harmful consequences on health and environment. 2,4-D mediating free radicals disturb the electrolytes homeostasis and Na^+/K^+-ATPase activity. The present study was designed to investigate the Selenium (Se) highly effective antioxidant potency in attenuating the 2, 4-D induced electrolytes alterations in experimental rats. The rats were arbitrarily categorized into 4 batches and treated for 28 consecutive days by oral route with 2, 4-D (150 mg/kg bw/day) associated or not with Se supplement (1 mg/kg bw/day). Results of the present study showed that 2,4-D mediating free radicals caused disruptions as indicated by the disrupted levels of erythrocytes Na^+/K^+-ATPase activity, intraerthrocytes sodium (Na^+), intraerthrocytes potassium (K^+), plasma sodium (Na^+), potassium (K^+), magnesium (Mg^{++}), calcium (Ca^{++}), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GSH reductase). However, supplementation with Se prevented the 2,4-D induced disruptions as indicated by restored levels of electrolytes, Na^+/K^+-ATPase activity and oxidative status. Overall, results indicate that Se supplementation may work as a potential antioxidant agent against oxidative damage in 2,4-D exposed animals by altering the ionic transport across the cell membrane.

Key words: 2,4-Dichlorophenoxyacetic acid, selenium, free radicals, electrolytes homeostasis, Na^+/K^+-ATPase.

INTRODUCTION

The rate of growth worldwide demand for agricultural commodities may prompt expanded utilization of chemical control items since these substances can contribute extensively in expanding yields and improving farm revenues. Pesticides, a broad group of biologically active compounds used for pest management, are among the most widely used chemicals worldwide (Benfeito *et al*., 2014). 2,4-Dichlorophenoxyacetic acid (2,4-D) is one among these pesticides. 2, 4-D is the first systemic phenoxy herbicide broadly applied against a wide range of leaf weeds in fields, lawns, parks and forest (Aquino *et al*., 2007). 2,4-D remains one of the most frequently used herbicides due to its economic value, good selectivity and broad-spectrum control of weed (Paszko *et al*., 2016). Its ubiquitous applications may enforce a substantial health hazard through skin contact, ingestion or inhalation (Suwalsky *et al*., 1996; Kurenbach *et al*., 2015; Islam *et al*., 2017). 2,4-D degradation leads to the production of chlorophenols or dioxins that exerts strong toxicity (Bukowska, 2003, Oghabian *et al*., 2014) by reactive oxygen species (ROS) generated free radicals. These free radicals are responsible for harmful effects on various biological molecules such as nucleic acids, lipids and proteins, thereby altering the normal redox status leading to increased oxidative stress (Mates, 2000; Bukowska, 2006; Phaniendra *et al*., 2015).

Membrane-bound enzymes play an essential role in the maintenance of erythrocytes normal functioning (Therien and Blostein, 2000). ROS generated free radicals can provoke degenerative modifications in red blood cells that can disturb the membranes' dynamic properties, fluidity and permeability and subsequently, membrane-bound enzymes activities. In fact, previous studies have well documented that elevation in free radical production and decrease in the GSH content caused membrane-bound enzyme, Na^+/K^+-ATPase inhibition which results in altered ion flux (Hazarika *et al*., 2001).

Selenium (Se) is an essential micronutrient embedded in several proteins and responsible for strengthening the defense mechanism of both human and animal (El-Demerdash and Nasr, 2014; Ventura *et al*., 2017). Se occurs in the form of the amino acid selenocysteine in various selenoproteins. Selenoproteins contribute in various biological processes including defense system, production of thyroid hormone and response of immune system (Ognjanovic *et*

al., 2008; Tinggi, 2008). Glutathione peroxidase (GSH-Px) selenoenzyme is responsible to prevent unlimited production of ROS in cells or inhibiting their interaction with other biomolecules (Weekley and Harris, 2013). Se provides the protection against free radical mediated damaging effects by the activation of this GSH-Px enzyme (Zoidi *et al*., 2018; Stanishevska, 2018).

Keeping previously reported studies in view, the present research was undertaken to evaluate the 2,4-D mediating free radicals effects on electrolyte homeostasis and Na^+/K^+-ATPase activity and to evaluate the ameliorative effects of Se against the 2,4-D induced electrolytes imbalances in experimental rats.

MATERIALS AND METHODS

Chemicals

Analytical grade (≥ 99%) chemicals including, 2,4-Dichlorophenoxyacetic acid (2,4-D) and Sodium Selenite (Na_2SeO_3) was purchased from Research Organics and Sigma Chemicals respectively.

Study design

Male Wistar rats (180-220g) were acquired from ICCBS (International Center for Chemical and Biological Sciences), University of Karachi. Animals were kept in polyethylene cages under controlled humidity (70%±50%) and a 12-h light–darkness cycle and habituated to housing condition for one week before initializing the experiment. The rat had free access to commercial pellet diet and water. The study for handling and caution of laboratory animals was conducted in compliance with the worldwide accepted guiding rules (Health research, extension act of 1985).

After the familiarization period, rats were indiscriminately allocated into four groups and treated for 28 consecutive days.

Group 1(n=10): Control group
Group 2 (n=10): 2,4-D group, treated with 2,4-D (150 mg/kg bw/day in deionized water) by oral route (Wafa *et al*., 2011).
Group 3 (n=10): 2,4-D+Se group, received both 2,4-D (150 mg/kg bw/day in deionized water) and Se supplement (1 mg/kg bw/day in deionized water) simultaneously by oral route.
Group 4 (n=10): Se group, treated orally with Se supplement (1 mg/kg bw/day in deionized water) (Kalender *et al*., 2015).

Sample collection

After 24 hours of the last dose of experimental group, rats were decapitated and samples of blood were collected in tubes coated with anticoagulant (heparin). The blood samples were then allowed to centrifuge at 2,200 g for 10 minutes to separate plasma and stored at -80°C until examination. Electrolytes estimation was done in freshly collected erythrocytes.

Estimation of Intraerthrocytes Na^+ and K^+

Erythrocytes were treated three times with magnesium chloride solution and aspirated the supernatant after every step of centrifugation as the earlier described method of Mayer and Starkey (1977). Final supernatant was then used for the assessment of Intraerthrocytes Na^+ and K^+ contents. Intensities were recorded by using Corning 410 flame photometer and units were expressed as mmol/L.

Erythrocyte membrane preparation

The washed erythrocytes were treated with Tris-HCl buffer (0.011M) three times. The membrane pellet was then allowed to re-suspend in Tris-HCl buffer (0.01M). The concentration of protein in the final membrane yield was estimated by Biuret method (Savory *et al*., 1968) and then refrigerated at -80^{o}C until the Na^+/K^+-ATPase activity was carried out.

Estimation of erythrocyte Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was assessed by the method of Raccah *et al*. (1996). Briefly, assay was incubated with and without 1mM Ouabain at 37^{o}C for 10 minutes. After incubation, 4 mM ATP was added to reaction mixture and subjected to incubate it again for 10 minutes, the reaction was subsequently stopped by adding trichloroacetic acid (TCA) and allowed to centrifuge for 10 minutes at 5,500 g at 4^{o}C. The obtained supernatant was used for the inorganic phosphate determination. Activity was expressed as inorganic phosphate released in nmol/mg of protein/hour.

Estimation of plasma electrolytes Na^+, K^+, Mg^{++} and Ca^{++}

Plasma electrolytes Na^+ and K^+ were estimated by using Corning 410 flame photometer (Mayer and Starkey, 1977). Mg^{++} level in plasma was assessed by the method of Hallry and Sky Peck (1964) and Ca^{++} by Ion Selective Electrode (ISEs) Jenway.

Estimation of malondialdehyde (MDA)

A lipid peroxidation indicator, MDA was assessed by the method of Okhawa *et al.* (1979). The principal of this procedure was based on measurement of thiobarbituric acid reacting substances (TBARS) which were formed as a byproduct of lipid peroxidation. The absorbance of pink color complex was recorded at 532 nm spectrophotometrically (Shimadzu UV-1900). Results were expressed as nmol/mL.

Estimation of superoxide dismutase (SOD)

SOD activity was assayed by the procedure of Kono (1978) as percent inhibition of the rate of nitroblue tetrazolium (NBT)-dye. The amount of NBT-dye colored was recorded spectrophotometrically at 560 nm. The activity was represented as U/mL.

Estimation of catalase (CAT)

CAT activity was determined by the method of Sinha (1972). Assay system containing dichromate/acetic acid solution and hydrogen peroxide produced chromic acetate upon heating at 100°C. The absorbance of chromic acetate produced was recorded spectrophotometrically 570 nm. The enzyme activity was represented as µmol/mL.

Estimation of glutathione reductase (GSH reductase)

GSH reductase activity was assessed by the method of Carlberg and Mannervik (1985). The assay system containing plasma, KH_2PO_4 buffer, reduced NADPH, and oxidized glutathione was thoroughly mixed and reaction was measured at 340 nm on kinetic spectrophotometer (PRIM 500) at 25°C for 5 minutes. Activity of GSH reductase was expressed as U/mL.

Statistical analyses

Data were shown as mean ± SD. Data of the present study were statistically analyzed by using Statistical Package Costat. One-way ANOVA was performed, and means were compared by Duncan Mutiple Range Test at $p < 0.05$.

RESULTS

Effects of 2,4-D and Se treatment on Na^+/K^+-ATPase activity and intraerthrocytes Na^+ and K^+ contents in control and treated rats

2,4-D administration showed a significant ($p < 0.05$) decrease in Na^+/K^+-ATPase activity in comparison with control group**.** However, in 2,4-D+Se group, Se treatment improved the Na^+/K^+-ATPase activity when compared with 2,4-D group ($p < 0.05$). In alone Se treated group, a noticeable elevated Na^+/K^+-ATPase activity was observed as compared with control group ($p < 0.05$). In contrast with control group, a remarkable increase in intraerthrocytes Na^+ content was observed in 2,4-D treated group ($p < 0.05$).While in remaining treated groups no significant change was noticed. Intraerthrocytes K^+ content was significantly ($p < 0.05$) decreased in 2,4-D exposed group as compared with control group. However, no remarkable increase in K^+ content of 2,4-D+Se group was noticed in contrast with 2,4-D treated group (Table 1)

Effects of 2,4-D and Se treatment on plasma electrolytes Na^+, K^+, Mg^{++} and Ca^{++} in control and treated rats

A significant ($p < 0.05$) decrease in plasma Na^+ level was observed in 2,4-D and 2,4-D+Se treated groups when compared with control group. However, in 2,4-D+Se group, an increase level of Na^+ was observed when compared with 2,4-D treated group ($p < 0.05$). On the other hand, a significant increase plasma K^+ level was observed in 2,4-D and 2,4-D+Se exposed groups ($p < 0.05$) in comparison with control group. However, no significant changes were observed when compared with 2,4-D exposed group**.** Plasma Mg^{++} level was significantly decreased ($p < 0.05$) in 2,4-D and 2,4-D+Se treated groups in comparison with control group. However, a significant increase in level of Mg^{++} was observed in 2,4-D+Se group as compared with 2,4-D group ($p < 0.05$). Plasma Ca^{++} level was reduced significantly in 2,4-D and 2,4-D+Se exposed groups in contrast with control group ($p < 0.05$). Conversely, no significant effects were observed in 2,4-D+Se group in comparison with 2,4-D treated group (Table 2).

Effects of 2,4-D and Se treatment on plasma oxidative stress biomarkers (MDA, SOD, CAT and GSH reductase) in control and treated rats

Plasma MDA levels in 2,4-D and 2,4-D+Se treated rats were found to be significantly increased ($p < 0.05$) when compared to control group. On the contrary, there was no significant difference observed in 2,4-D+Se group in MDA level when compared to 2,4-D alone group. Activity of SOD was significantly declined in 2,4-D and 2,4-D+Se groups in comparison with control rats ($p < 0.05$). In contrast with 2,4-D exposed group SOD activity of 2,4-D+Se group was improved significantly ($p < 0.05$). CAT activity was found to be decreased in 2,4-D and 2,4-D+Se exposed groups when compared with control ($p < 0.05$). But, CAT activity was considerably increased in 2,4-D+Se group when compared to control ($p < 0.05$). GSH reductase activity was found to be remarkably decreased in 2,4-D and 2,4-D+Se exposed group than control rats ($p < 0.05$). While, supplementation with Se significantly restored the decrease GSH reductase activity of 2,4-D+Se group when compared to 2,4-D alone ($p < 0.05$). In alone Se treated group, a remarkable increase in GSH reductase activity was observed in comparison with control group ($p < 0.05$) (Table 3).

Table 1. Effects of 2,4-D and Se treatment on erythrocytes Na^+/K^+-ATPase activity and Intraerthrocytes Na^+ and K^+ contents in control and treated rats.

Parameters	Control	2,4-D	2,4-D+Se	Se	$LSD_{0.05}$
Na^+/K^+-ATPase activity (nmol/mg of protein/hour)	276.06 ± 52.36 b	183.57 ± 50.12 c	270.69 ± 57.05 b	340.55 ± 76.12 a	54.235
Intraerthrocytes Na^+ (mmol/L)	7.81 ± 0.23 b	8.51 ± 0.34 a	7.83 ± 0.87 b	7.40 ± 0.78 b	0.565
Intraerthrocytes K^+ (mmol/L)	130.00 ± 12.34 a	105.80 ± 10.33 b	126.09 ± 10.88 a	134.11 ± 10.89 a	10.105

Values are represented as mean ± SD, (n=10); LSD = Least Significant Difference; similar letters in a row are non-significant according to Duncan Multiple Range Test at $p < 0.05$.

Table 2. Effects of 2,4-D and Se treatment on plasma electrolytes Na^+, K^+, Mg^{++} and Ca^{++} in control and treated rats.

Parameters	Control	2,4-D	2,4-D+Se	Se	$LSD_{0.05}$
Na^+ (mmol/L)	138.80 ± 1.62 a	132.50 ± 1.51 c	137.20 ± 1.22 b	139.41 ± 1.95 a	1.450
K^+ (mmol/L)	6.51 ± 0.21 b	7.58 ± 0.31 a	7.31 ± 1.16 a	6.16 ± 0.59 b	0.6156
Mg^{++} (mg/dL)	2.70 ± 0.38 a	1.68 ± 0.22 c	2.21 ± 0.32 b	2.78 ± 0.19 a	0.2629
Ca^{++} (mg/dL)	3.88 ± 0.39 a	2.60 ± 0.69 b	2.95 ± 0.26 b	3.70 ± 0.59 a	0.467

Values are represented as mean ± SD, (n=10); LSD = Least Significant Difference; similar letters in a row are non-significant according to Duncan Multiple Range Test at $p < 0.05$.

Table 3. Effects of 2,4-D and Se treatment on plasma oxidative stress biomarkers (MDA, SOD, CAT and GSH reductase) in control and treated rats.

Parameters	Control	2,4-D	2,4-D+Se	Se	$LSD_{0.05}$
MDA (nmol/mL)	2.22 ± 0.40 c	3.47 ± 0.23 a	2.57 ± 0.31 a	2.15 ± 0.15 c	0.262
SOD (U/mL)	148.10 ± 5.36 a	72.65 ± 11.08 c	112.50 ± 7.91 b	142.31 ± 18.99 a	10.872
CAT (µmol/mL)	600.50 ± 20.06 a	362.80 ± 40.80 c	460.45 ± 76.49 b	610.50 ± 49.35a	46.148
GSH reductase (U/mL)	0.135 ± 0.021 b	0.063 ± 0.006 d	0.114 ± 0.008 c	0.151 ± 0.012 a	0.011

Values are represented as mean ± SD, (n=10); LSD = Least Significant Difference; similar letters in a row are non-significant according to Duncan Multiple Range Test at $p < 0.05$.

DISCUSSION

Phenoxyacetic acid herbicide 2,4-D and its derivatives have been the most widely used herbicides in all over the world. Their popularities among farmers and other users are undeniable. Despite their sophisticated contributions, they had caused a lot of problems to the environment. It is highly concern for their strong genotoxic effects in different types of organisms (Garabrant and Philbert, 2002). Trace mineral in the form of Se is associated with specific selenoproteins which are known to act as a negotiator in suppressing the carcinogenesis (El-Bayoumy *et al*., 1992).

Chlorinated phenol acts as the main precursor in chlorophenoxy herbicides production (Saracci *et al*., 1991). In fact, the inhibitory effects of chlorophenoxy acids and their metabolites on Na^+/K^+-ATPase could be one of the underlying biochemical mechanisms that leads to cellular dysfunctions (Duchnowicz *et al*., 2005). Several research evidences proposed that phenoxyl herbicides can perturb cell membrane structure and function by incorporating into them. In the current study, we observed the decreased levels of Na^+/K^+-ATPase in 2,4-D exposed group when compared with the control group (Table 1). This change of Na^+/K^+-ATPase activity could be due to erythrocyte membrane modification to a great extent in composition and fluidity (Bukowska, 2003; Vasic *et al*., 2008). The decline activity of ATPase consequently affects the membrane permeability and gradients (Francescato *et al*., 2010). Likewise, in current research, it was found that decline activity of ATPase as a result of 2,4-D exposure disrupted the membrane gradient as manifested by increased intraerythrocytes Na^+ (Table 1) and decreased intraerythrocytes K^+ contents (Table 1) and in plasma its vice versa (Table 2). These electrolytes imbalance played a key function in toxicity development of 2,4-D exposed group (Noori and Mahboob, 2012).

Na^+/K^+ ATPase play a key role to maintain the active transport of K^+ intracellularly during the action potential. Mg^{++} is required for this Na^+-K^+ pump because ATP binds with $Mg^{++}$to make it biologically active (Parikka *et al*., 1999). In the case of Mg^{++} depletion, the pump function is impaired which consequently reduced the activity ATPase by insufficient pumping of K^+ into the cell (Bianchetti *et al*., 1990). In present findings, decrease Mg^{++} levels of plasma (Table 2) due to 2,4-D exposure is responsible for electrolytes disturbances. Therefore, the potassium gradient cannot be maintained. Additionally, a remarkable decline in Ca^{++} level was observed in 2,4-D exposed group (Table 2). Ca^{++} helps in regulating metabolic reactions by invigorating or repressing the key enzymes and sustaining Na^+/K^+ ATPase in an active state of resting potential. Elevated intracellular Ca^{++} levels shuts down Na^+/K^+ ATPase (Gloy *et al*., 1999).

There are numerous experimental researches that explained the vitality of Se as an anti-carcinogenic negotiator (Rotruck *et al*., 1972; Dumas *et al*., 1990; Borges *et al*., 2004; Soudani *et al*., 2010). The exact mechanism of exerting salts effectiveness to provide protection is unclear; nevertheless, a number of assumptions have been suggested regarding its protective effects. From literature reviews, it is well established that sodium instead of selenite is accountable for this protection. It exerts its protective effect by the certain amendment in osmolality of extracellular fluid, which consequently alters stress response (Vermeulen *et al*., 1993). Our study outcomes elucidated the Se role against 2,4-D induced electrolytes disruptions which might be associated with stress response by Se salt loading that induced hydration (Ogilvie *et al*., 1988). This hydration provides an evidence for further considerations on the underlying mechanism.

Lipid peroxidation is implicated in pesticide induced toxicity development (Akhgari *et al*., 2003). In the present findings, elevated levels of MDA (Table 3) following 2,4-D exposure was probably credited to the excessive production of ROS that leads to develop modifications in the structure of molecules and lastly cell damage (Celik *et al*., 2006). The detoxification of ROS involves the co-operative action of all intracellular antioxidant enzymes including SOD, CAT and GSH-Px that eradicate free radicals produced by ROS, consequently providing protection to cells. Any harm to these enzymes, could notably influence the defensive system (Rana *et al*., 2002; Halliwell and Gutteridge, 2015). In the current study, 2,4-D intoxication depleted the antioxidant enzymes activities including SOD, CAT and GSH reductase (Table 3) which indicated the failure of antioxidant defense system to overcome the influx of ROS induced by 2,4-D.

In the current study, Se treatment showed imperative consequences concerning MDA levels and antioxidant enzymes disruptions which were generated by 2,4-D exposure (Saito and Takahashi, 2002; Letavayova *et al*., 2006). The incorporation of selenocysteine in selenoproteins (GPx and thioredoxin reductase) was responsible for reducing the highly reactive radical induced oxidative damage by scavenging or counterbalances ROS (Rotruck *et al*., 1973; Toufektsian *et al*., 2000).

Conclusion

Present findings in this study suggest that Na^+/K^+-ATPase activity impairments and alterations in the electrolytes and oxidative stress biomarkers provoked by 2,4-D play a vital function in toxicity development. Thus,

supplementation with nutritional trace element Selenium could be a useful therapeutic option through the strengthening of endogenous antioxidative systems associated with electrolytes disturbance leading to cellular toxicity induced by 2,4-D.

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