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**RESEARCH ARTICLE**

# BIOCHEMICAL STUDY ON THE ROLE OF SODIUM BUTYRATE IN PROTECTION AGAINST OXIDATIVE STRESS INDUCED BY PARAQUAT

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**ABSTRACT**

Paraquat (PQ) is used as herbicide. The present study was designed to investigate the potential protective effects of sodium butyrate (SB) against the toxic effects induced by PQ. The experiment was carried out on 50 male albino rats divided into five groups. Some parameters were assessed. The results revealed that PQ induces significant increase in malondialdehyde levels, serum 8-OHdG and nuclear histone deacetylase (HDACs) activity. Cytoplasmic GSH concentration and catalse activity were decreased in a significant way. Serum and tissue TAC remained unchanged. SB administration in both co-treated and post-treated rats reversed the oxidative damage induced by PQ as documented by the significant decrease in serum, tissue malondialdehyde and serum 8-OHdG. Nuclear GSH, HDACs and CAT activity were elevated in co-treated and post-treated groups. In conclusion, sodium butyrate is able to rescue the redox machinery and control the intracellular ROS balance thus switching off oxidative damage induced by PQ.



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## INTRODUCTION

Paraquat (PQ) is widely used as herbicide and considered as a very dangerous pollutant of the environment, as it readily binds to both clay and organic matter in soil and is very slowly biodegraded. It is not volatile and its contamination resulted in a number of health effects. It is distributed quickly by blood to reach all organs and tissues (Abdel-Hady and Abdel-Rahman, 2013). The intentional and accidental poisoning of PQ is a major public health issue in developing countries. Ingestion of even a small amount of PQ could lead to a fatal outcome (Chen *et al.,* 2013). It is highly lethal upon entry into human body with the lungs being its major target organ. After entering the alveolar epithelial cells, PQ undergoes oxidative reaction and subsequently triggers the reactive oxygen cascade. The numerous reactive oxygen species (ROS) released from the cascade oxidize the unsaturated fatty acids in various biomembranes, result in damages of the capillary endothelial and alveolar epithelial cells, thus leading to pulmonary injury in addition to damaging the cellular membrane lipid (Meng *et al.,* 2013). Previous studies indicated that prenatal exposure to pesticides including PQ may develop certain disorders such as Parkinson's disease (PD) and leukemia in offspring (Monge *et al.,* 2009). An increasing amount of evidence suggests that oxidative stress is linked to either the primary or secondary pathophysiologic mechanisms of multiple acute and chronic human diseases. Cell death in neuronal tissues has been implicated in numerous neurodegenerative diseases including stroke and Alzheimer's (McCarthy *et al.,* 2004; Klaunig and Kamendulis, 2004).

Butyric acid is a product of bacterial fermentation of carbohydrates in the rumen of multigastric animals and in the colon of omnivores, such as humans (Hinnebusch *et al.,* 2001). The induction of apoptosis of tumor cells by butyrate is thought to be an important mechanism in the natural protection against colorectal cancer; since one of the major actions of butyrate is to inhibit histone deacetylase (HDACs). Sodium butyrate (SB) is known to modulate gene expression in the endothelium (Safaya *et al.,* 2009) and induce apoptotic effects in a number of cancers and up-regulate pro-apoptotic molecules in cancer cells (Mu *et al.,* 2013). Moreover, SB has been focused on since it possesses anti-inflammatory activities, but its exact mechanisms of action are not well understood. Since SB inhibits HDAC resulting in the relative hyperacetylation of core histone proteins (H3 and H4), it probably regulates the expression of some inflammatory related genes (Machado *et*

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*al.,* 2012). The aim of this study is to assess the protective role of SB as an antioxidant against the oxidative damage induced by PQ in rats.

## MATERIALS AND METHODS

### Animal groups

This study was carried out on 50 male albino rats (*Sprague dawley*), their weight ranged between 150-175 g. The experimental protocol was approved by Tanta University Ethics Committee and Animals Research. The rats were randomly and equally divided into five groups (10 animals each). The first group was the negative control group (GpI) in which animals were injected with only saline. The second was the positive control group (GpII) which included I.P. injected rats with PQ in a dose of 10 mg/kg body weight once aweek for 6 weeks to induce oxidative stress (Muthukumaran *et al.,* 2014*)*.The third group (GpIII) comprised rats injected by sodium butyrate in a dose of 100 mg/kg body weight I.P (Sun *et al.,* 2013) twice aweek for 6 weeks. The fourth group (GpIV) was the co-treated group in which rats simultaneously I.P injected with both PQ in a dose of 10 mg/kg once aweek and with 100 mg/Kg of SB twice a week for 6 weeks. The third group (GpV) was the Post-treatment group in which rats I.P injected with 10 mg/Kg PQ once aweek for 6 weeks. Then, they were administered 10 mg/kg sodium butyrate twice a week for another 6 weeks.

### Biochemical analysis

At the end of the experiment, the animals were sacrificed under anesthesia by decapitation and blood samples were taken. Serum was analyzed to determine malondialdehyde (MDA) colormetrically using thiobarbituric acid (TBA), trichloroacetic acid (TCA) and n-butanol (Sigma-Aldrich St. Louis MO, USA), according to Ohkawa *et al.* (1979). Total Antioxidant Capacity (TAC) determined by commercial kit (Biodiagnostic, TA2513, Egypt) according to Samir *et al.* (2012) and serum Oxidative DNA damage (8-OHdG) using ELISA kit (Enzo-life sciences, ADI-EKS-350, USA) according to the method of Rangel-Lopez *et al.* (2013). Brain homogenates were prepared as 10% (w/v) homogenate by weighing two pieces of each specimen and homogenized with a Potter-Elvenhjem tissue homogenizer (20-30 up and down strokes). One part was homogenized in cold phosphate buffer saline 5 mM (pH 7.4) and the other one was homogenized in cold potassium phosphate buffer 10 mM (pH 7.4). Homogenates were centrifuged at 7,700 *x g* for 30 minutes at 4$^{o}$C, and the resultant supernatant (free of insoluble materials) was assayed for estimation of MDA, TAC, total protein content (Breikaa *et al.,* 2013) and the other part used to get nuclear extract using nuclear extraction kit (BSP002, Biobasic INC, Canada) according to Ouyang *et al.* (2009).The HDAC activity was assessed by Colorimetric HDAC Activity Assay Kit (Biovision, K331 USA) according to Li *et al.* (2012). Reduced glutathione (GSH) and catalase (CAT) were analyzed in the cytoplasmic extract. GSH was analyzed using Disodium hydrogen phosphate solution and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) according to method of Ellman (1959). CAT was determined by Phosphate buffer pH 7.0 and hydrogen peroxide ($H_2O_2$) according to Xu *et al.* (1997).

### Statistical analysis

Statistical analysis was performed using Graphpad prism 6 software (Graphpad, San Diego, CA). Data (n=10) were expressed as mean values±SEM. All data were subjected to one-way ANOVA test to determine differences among groups, followed by Tukey's post hoc test. Statistical significance was taken at the $P < 0.05$ level.

## RESULTS

DNA damage and lipid peroxidation were determined in serum as 8-OHdG and MDA.8-OHdG and MDA significantly increased by PQ injection (GpII). Co and post administration of SB (GpIV and GpV) was able to restore 8-OHdG and MDA back to their nearly normal levels. On the other hand, TAC levels didn't show any significant change in all groups (Table 1). Nuclear HDACs activity and MDA levels in the brain significantly increased by PQ injection (GpII). There was no significant change in the TAC levels in the brain tissues. Co-administration (GpIV) and post administration of SB (GpV) showed good improvement in brain MDA level but nuclear HDACs activity still high (Table 2). PQ injected rats (GpII) showed significantly decrease in CAT activity and GSH concentrations in the cytoplasmic extract of the brain. SB administration (GpIV and GpV) showed good improvement in the activity of CAT and GSH content (Table 3).

There was significant positive correlation between brain MDA, serum MDA and 8-OHdG levels. However, there was no correlation between both serum, tissue TAC and both MDA in brain and serum 8-OHdG level. There was also no correlation between the activity of HDACs and serum 8-OHdG, brain MDA and serum MDA. Moreover, there was no correlation between HDACs activity and both serum and brain TAC level. Brain GSH and CAT had positive significant correlation. The results revealed negative significant correlation between serum MDA and both brain GSH level and brain CAT level. MDA level in brain showed negative significant correlation with both GSH level and CAT activity in brain. There was a negative significant correlation between serum 8-OHdG level and both GSH content and CAT activity in the brain (Table 4).

## DISCUSSION

The results showed that PQ administration in rats (GpII) lead to oxidative DNA damage represented in dramatically significant increase in 8-OHdG levels (reached to about 2.5 folds). While butyrate only injected rats showed 8-OHdG levels within the normal values of the control rats. Butyrate co-treated and post treated PQ injected rats (GpIV and GpV) showed good regression to nearly normal values especially the post treated rats. These results are in agreement with previous studies which reported that 8-OHdG immunereactive cells in hippocampus and substantianigra increased significantly after PQ administration as compared to the control (Wu *et al*., 2013) and in the hippocampus of mice after PQ administration (Chen *et al*., 2010). This DNA damage effect of PQ can be discussed

according to the explanation that when PQ is absorbed into the body, ROS is produced to attack DNA bases and in turn to form a variety of modified bases such as 8-hydroxyguanine, 8-hydroxyl adenine, cytosine glycol, and thymine glycol. Guanine molecules is the most susceptible since it contain high orbital energy, resulting in accelerating generation of 8-OHdG (Zhang *et al.,* 2011). While the effect of butyrate can be explained by the chemical structure of butyrate makes the simple action as scavenger improbable, the effects of butyrate on the chromatin structure and on the DNA repair mechanisms are known (Bingham, 1997). MDA is one of the most popular and reliable markers that determine oxidative stress in clinical situations (Giera *et al.,* 2012). MDA appears to be the most mutagenic product of lipid peroxidation. The results showed

**Table 1. Oxidative stress biomarkers in serum**

| Group (treatment) | MDA(nmol/ml ) | TAC (µmol /ml) | 8-OHdG (ng/ml ) |
|---|---|---|---|
| GpI (saline) | 11.32± 0.176 | 2.22±0.168 | 4.517±0.414 |
| GpII (PQ) | 17.21±0.178 [a] | 1.81±0.144 | 11.419±0.428 [a] |
| GpIII (butyrate) | 11.59±0.131 | 2.13±0.159 | 4.97±0.221 |
| GpIV (co PQ+SB) | 12.24±0.274[b] | 1.91±0.101 | 5.277±0.427 [b] |
| GpV (post PQ+SB) | 11.78±0.154[b] | 2.01±0.104 | 4.746±0.256 [b] |

*Data are expressed as mean±SEM of 10 observations.The symbols (a) and (b) indicate a significant changes ($p<0.05$) in comparison with GpI (negative control) and GpII (Paraquat administered group), respectively.

**Table 2: Oxidative stress biomarkers in brain tissue**

| Group (treatment) | MDA(nmol/g.tissue ) | TAC (mol /g.tissue) | Nuclear HDACs activity (O.D/mg.protein ) |
|---|---|---|---|
| GpI (saline) | 56.18±1.5 | 1.13±0.04 | 1.62±0.092 |
| GpII (PQ) | 98.96±1.79[a] | 0.99±0.06 | 3.62±0.34[a] |
| GpIII (butyrate) | 57.12±1.63 | 1.12±0.03 | 1.99±0.098 |
| GpIV(co PQ+SB) | 61.8±0.91[b] | 0.98±0.028 | 3.72±0.14[a] |
| GpV(post PQ+SB) | 58.74±1.10 [b] | 1.01±0.023 | 4.11±0.15[a] |

*Data are expressed as mean±SEM of 10 observations. The symbols (a) and (b) indicate a significant changes ($p<0.05$) in comparison with GpI (negative control) and GpII (Paraquat administered group), respectively.

**Table 3: Oxidative stress biomarkers in the brain's cytoplasmic extract**

| Group (treatment) | GSH (mg/g. tissue) | CAT (mg/min/g.tissue) |
|---|---|---|
| GpI (saline) | 7.60±0.176 | 19.2±0.515 |
| GpII (PQ) | 3.39±0.112[a] | 5.85±0.336[a] |
| GpIII (butyrate) | 7.90±0.167 | 18.78±0.509 |
| GpIV(co PQ+SB) | 5.79±0.167[b] | 11.68±0.262 [b] |
| GpV(post PQ+SB) | 5.94±0.126[b] | 12.18±0.399 [b] |

*Data are expressed as mean±SEM of 10 observations. The symbols (a) and (b) indicate a significant changes ($p<0.05$) in comparison with GpI (negative control) and GpII (PQ administered group), respectively.

**Table 4: Correlation matrix of all studied parameters of all groups**

| | | Serum MDA | Brain MDA | Serum 8-OHdG | Brain GSH | Brain CAT | Serum TAC | Brain TAC |
|---|---|---|---|---|---|---|---|---|
| **Serum MDA** | R | | | | | | | |
| | P-value | | | | | | | |
| **Brain MDA** | R | 0.946 | | | | | | |
| | P-value | <0.001 | | | | | | |
| **Serum 8-OHdG** | R | 0.908 | 0.906 | | | | | |
| | p-value | < 0.001 | <0.001 | | | | | |
| **Brain GSH** | R | -0.859 | -0.835 | -0.787 | | | | |
| | P-value | <0.001 | < 0.001 | < 0.001 | | | | |
| **Brain CAT** | R | -0.779 | -0.795 | -0.700 | 0.923 | | | |
| | P-value | < 0.001 | < 0.001 | < 0.001 | < 0.001 | | | |
| **Serum TAC** | R | -0.274 | -0.211 | -0.2956 | 0.3442 | 0.3456 | | |
| | P-value | 0.055 | 0.192 | 0.0874 | 0.0144 | 0.0140 | | |
| **Brain TAC** | R | -0.241 | -0.352 | -0.1832 | 0.4074 | 0.4929 | 0.181 | |
| | P-value | 0.0922 | 0.0123 | 0.203 | 0.0033 | <0.001 | 0.208 | |
| **Nuclear HDACs** | R | 0.353 | 0.303 | 0.281 | 0.357 | 0.477 | -0.262 | -0.312 |
| | P-value | 0.012 | 0.0326 | 0.0485 | <0.001 | <0.001 | 0.066 | 0.0275 |

highly significant increase in MDA levels in GpII (rats injected with PQ) in both serum and brain homogenate (reached to about 1.5 and 1.7 folds, respectively) compared to the control rats. While after butyrate (co and post) treatment (GpIV and GpV), MDA levels returned back to nearly normal concentrations. These results is logic because PQ itself is an oxidant as it forms a PQ radical that transfers its extra electron to an oxygen molecule generating a superoxide anion such a superoxide anion gets converted to hydrogen peroxide that subsequently turns into either a harmful hydroxyl radical or is directly detoxified by antioxidant enzymes. Hydroxyl radicals along with other free radicals react with polyunsaturated fatty acids to yield lipid hydro-peroxides. These products initiate the lipid radical chain reaction leading to oxidative damage (Prakash *et al.,* 2013). In agreement with the present study, previous studies indicated MDA increasing levels in different parts of the brain especially in nigrostriatalporion (Yadav *et al.,* 2013) and in hippocampus, pyramidal neurons and cerebral cortex (Djukic *et al.,* 2012). Concomitantly, various studies reported the increased levels of MDA in serum of rats over intoxicated with PQ (Sikoraandbodziarczyk, 2013; Abdel-Hady and Abdel-Rahman, 2013; Meng *et al.,* 2013; Khodayar *et al.,* 2014).

The protective effect of SB can be discussed according to its role as an anti-inflammatory and anti-oxidative stress agent, primarily *via* inhibition of nuclear factor κB (NF-κB) activation, which may result from the inhibition of HDAC. Many studies proved this antioxidant effect in both brain tissue and serum (Courtois *et al.,* 2003; Steckert *et al.,* 2013). A possible mechanism of action for the neuroprotective effects of SB is the up-regulation of brain-derived neutrophic factor (BDNF) which promotes survival of nerve cells. It has been demonstrated that HDAC inhibitors, sodium butyrate, up-regulate BDNF expression in astrocytes and protect dopamine neurons (Wu *et al.,* 2008). From the present data, it has been shown that there is very significant positive correlation between brain and serum MDA. It can be concluded that the membranes of brain cells and tissues were severely damaged and the massive increase in brain MDA was sufficient to appear more clearly in the serum. This highly significant correlation suggested that measuring serum MDA is a perfect alternative to tissue MDA and is effort and time saver.

TAC could be a reliable biomarker of diagnostics and prognostics values for many diseases (Kusano and Ferrari, 2008). In this study, TAC concentrations didn't show any significant change in both serum and homogenate in all treated rats in comparison with the control. These results can be explained by different ways. Some studies indicated that TAC may have been chemically appropriate in the context of the in vitro but not in vivo antioxidant enzyme activities in plasma (Sies, 2007). Regulation of the capacities of antioxidant in response to changing levels of oxidative stress is a prerequisite for efficient defense (Tong *et al.,* 2006). Moreover, the antioxidant system is highly complex, and synergistic and antagonistic interactions between individual antioxidants make it challenging to draw inference regarding overall antioxidant capacity from information about the levels of multiple individual antioxidants (Costantini, 2008), yet without sufficient levels of other antioxidants, it can itself act as a powerful pro-oxidant and cause extensive damage to lipids (Bowry *et al.,* 1992). In the present study, SB was used as a protective antioxidant however its impact showed no statistically significant difference and this may be attributed to insufficient level of the other antioxidants. TAC assays do not measure total antioxidant capacity. In general, they measure predominantly the low molecular weight, chain breaking antioxidants, excluding the contribution of antioxidant enzymes and metal binding proteins (Young, 2001). TAC does not mean all the antioxidants in the defense system in vivo, but it means the capacity of free radical scavenging by the radical scavenging antioxidants contained in the test sample (Serafini and Del-Rio, 2004). It should be noted, however, that TAC values measured are semi-quantitative at best and do not distinguish reactivity and concentration. More importantly, it should be kept in mind that they do not necessarily correlate with the capacity of antioxidation or inhibition of oxidation (Niki, 2010). Consistent with the present study, there is no significant difference between the studied groups in the total antioxidant capacity in plasma and in rat brain regions, frontal cortex and the hippocampus (Firuzi *et al.,* 2006; Pomierny *et al.,* 2014). There was no correlation between both serum and tissue TAC and other parameters under study. So, it has been demonstrated that using TAC as a biomarker of oxidative insults is not decisive and more full-scale methods are suggested for the assessment of enzymatic antioxidants.

GSH is a vital constituent of cells throughout the body, acting as a redox buffer, and as cofactor for signal transduction, antioxidant defense, and electrophile defense, especially in the brain (Johnson *et al.,* 2012). The current work emphasized that PQ was a spectacular agent in inducing the oxidative damage as was demonstrated by the significant decrease in the concentration of the GSH in the tissues of brain in PQ group to reach to about 44 and 42% of its levels as compared to the control and SB groups, respectively. However, the co and post administration of SB showed good improvement represented in increasing brain GSH levels in GpIV and GpV. It can be explained Building on the role of PQ as oxidative agent, its redox cycling rapidly oxidizes NADPH. NADPH is responsible for regeneration of GSH from GSSG. This has been postulated to lead to secondary changes on cellular metabolism and impairs defenses against ROS by decreasing glutathione level (Gawarammana and Buckley, 2011). In agreement with the current work, Djukic *et al.* (2012) documented the depletion of GSH content along with the increased activity of GPx in the vulnerable brain regions and this was recognized as the most responsive biomarkers of the early stage of PQ induced neurotoxicity. Kang *et al.* (2009) elucidated that I.P injection of PQ increased the oxidized glutathione and decreased the reduced glutathione in the brain tissue. It can be proposed that repeated PQ injection induce dopaminergic neurotoxicity through generation of oxidative stress while administration of SB increases expression of DJ-1 protein. DJ-1 protein is responsible for eliminating metabolic insults including stress response through distinct independent mechanisms. One of these mechanisms involve increasing cellular GSH levels through selective upregulation of the rate limiting step in GSH synthesis-glutamate cysteine ligase (GCL) (Zhou *et al.,* 2011). Sharma and Sharma (2013) explained the role of SB in recovering induced ROS and restoring GSH content in rat brain

tissues. CAT as an antioxidant enzyme protects the cells from oxidative stress and inactivates many environmental mutagens. The present study elucidated the significant decrease in CAT activity after PQ administration as compared to both the control and SB groups. It can be discussed according to the role of nitric oxide; an important secondary mediator of several biological functions significantly increases in PQ exposed mice, causing formation of peroxynitrite, a potent oxidant, which attacks a wide range of biological targets including CAT (Yadav *et al.,* 2013). Other studies showed decreased level of CAT activity in brain tissues after PQ intoxication (Gupta *et al.,* 2010; Kumar *et al.,* 2010). In the co-treatment and post-treatment groups, SB ameliorated the PQ induced stress and improved the CAT activity in the tissues of brain. The mode of action of butyrate depends on its proposed antioxidant properties and its ability to affect intracellular enzymes such as CAT and SOD responsible for reduction of $H_2O_2$ (Rosignoli *et al.,* 2001).

From the data represented in the current study, there was positive significant correlation between CAT activity and GSH content which mean that the body of rats responded to oxidative damage by consuming GSH in favor of removing stress but the stress was severe enough to destroy CAT enzyme. Additionally, both CAT and GSH showed significant negative correlation with serum 8-OHdG and both brain and serum MDA. These results illustrated that the relation between oxidative stress and antioxidant system changes according to the severity of stress and the ability of body to overcome it.

Acetylation and deacetylation of the histone-tail play a pivotal role in the epigenetic regulation of gene expression and many other cellular events, including growth, differentiation, development, learning and memory, and apoptosis (Abel and Zukin, 2008). Nuclear HDACs activities in the present study demonstrated extremely significant increase in rats injected with PQ (GpII); this increase reached to more than 2 folds. While in co-treated rats with SB (GpIV) and SB post-treated rats (GpV), SB unexpectedly did not show inhibitory effect on HDACs activities. Moreover, HDACs activities remained elevated compared to the control group and in parallel with the PQ administered rats (GpII). There is more than one possible explanation for these results (Peterson, 2002). Firstly, the majority of HDAC inhibitors as SB target multiple isoforms of the classic HDAC family (classes I, II, and IV) but do not inhibit SIRT family members. Short-chain fatty Acids, these inhibitors include compounds with rather simple structures, such as valproic acid, phenyl butyrate, and butyrate (Grayson *et al.,* 2010). SB has diverse properties and its clinical development has been hampered by its short half life and difficulty in achieving millimolar levels in vivo (Foglietta *et al.,* 2014). Secondly, over-expression of histone deacetylase 1 confers resistance to SB through a p53-mediated pathway. HDAC1 has antagonistic effect for SB (Bandyopadhyay and Mishra, 2004). Another study also showed that over-expression of all three members of the class I HDAC family HDAC1, HDAC2, and HDAC3 reduced the potency of SB. Moreover, both HDAC6, HDAC10 also showed resistance to SB due to their unique structure (Guardiola and Yao, 2002). The current work demonstrated that there was no correlation between the activity of HDACs and serum 8-OHDG, brain MDA and serum MDA. There was also no correlation between HDACs activity and both serum and brain TAC level. Moreover, the results demonstrated that there was no correlation between the activity of HDACs and both the activity of brain catalase and brain GSH content. These data had proposed explanation; the oxidative damage was intensive enough to let the activity of HDACs remain elevated in the co-treatment and post-treatment groups in order to alleviate this stress. However, at the same time the ROS resulted in the decrease of both CAT and GSH in the brains of rats and after treatment by SB their level was improved.

### Conclusion

In conclusion, PQ induces oxidative stress. To ameliorate this oxidative damage, HDACs activity is increased to counteract cellular stress and DNA damage. The inhibitory effect of SB was masked because its effect was resisted by some HDACs. It was unable to inhibit some groups of HDACs such as the class III HDACs. Additionally, SB was able to increase the activity of some HDACs such as HDAC-5. These results explain the diverse action of SB on HDACs. Different HDACs are regulated differently by HDAC inhibitors, suggesting differential sensitivity and roles for the individual enzymes.

### Recommendation

Further investigations may be needed to elucidate the role of short-chain fatty acids as antioxidants in alleviating oxidative stress. The role of other HDACIs in protecting brain tissues from induced damage may need more studies.

### Abbreviations

Paraquat= PQ, Sodium Butyrate= SB, Histone deacetylases= HDACs, Reduced glutathione= GSH, Reactive Oxidative Stress= ROS, 8-hydroxy-2-deoxy-guanosine= 8-OHdG, Total Antioxidant Capacity= TAC, Catalase= CAT, Malondialdehyde= MDA.

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