Influence of Vitamin C on phenytoin induced haematotoxicity and oxidative stress in rats

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ABSTRACT
Phenytoin is used widely in the treatment of epilepsy. The drug produces haematological toxicities such as thrombocytopenia, leukopenia, neutropenia, agranulocytosis, pancytopenia, hemolytic anemia and aplastic anemia. The arene oxide metabolites of phenytoin are expected to cause stem cell death leading to hematological disturbances via oxidative stress. The objective of the study is to investigate the dose dependent effect of vitamin C supplementation on oxidative stress and haematotoxicity induced by chronic administration of phenytoin (20 mg/kg, p.o) for a period of 45 days. On 45th day of phenytoin and phenytoin plus vitamin C treatment, blood was withdrawn from retro orbital plexus of rats and was subjected to estimation of hematological parameters, enzymatic, non enzymatic antioxidants, total antioxidant status and lipid peroxidation. Vitamin C supplementation significantly increased the total RBCs, haemoglobin, total WBCs, platelets and packed cell volume which was decreased by phenytoin, in a dose dependent manner. Vitamin C improved the activities of SOD, catalase and augmented the levels of reduced glutathione, vitamin C, total antioxidants and thereby reversed the oxidative stress induced by phenytoin. Vitamin C prevented oxidative stress and hematologic impairment induced by phenytoin possibly through its ability to scavenge reactive oxygen species. These findings are of interest in view of the high prevalence of hematological side-effects and oxidative stress associated with the long term phenytoin therapy.

Key Words: Phenytoin, ascorbic acid, oxidative stress, antioxidants, haematotoxicity.

INTRODUCTION
Epilepsy is one of the most common neurological diseases, affecting approximately 1% of population1. At a conservative estimate, 50 million people have epilepsy world wide. The annual incidence ranges from 20-70 cases per 1,00,0002 and the point of prevalence is 0.4-0.8%.3 The antiepileptic drugs account for about 4.5 – 11.5% cases of aplastic4, megaloblastic5, haemolytic anaemia7 and thrombocytopenia. Phenytoin, a hydantoin anticonvulsant is used widely in the treatment of generalized or partial seizures except absence seizures. The drug produces haematologic toxicities such as thrombocytopenia8,9,10, leukopenia11, neutropenia, agranulocytosis12,13,14, pancytopenia and rarely, hemolytic anemia, aplastic anemia4 and pure red cell aplasia5,15. The arene oxide metabolites of phenytoin covalently bind to macromolecules, thereby directly cause stem cell death leading to aplastic anemia, toxicity to lymphocytes16, 17 and depressed cellular as well as humoral immunity18. Phenytoin reduced serum glutathione concentration and increased lipid peroxidation in humans19. Phenytoin also reduced the total antioxidant capacity of sera of epileptic patients20. Sobaniec, et. al., (2007) reported that long-term treatment with phenytoin reduced activities of endogeneous antioxidant enzymes like superoxide dismutase, glutathione reductase, glutathione peroxidase and non enzymatic antioxidants like Vitamin C, Vitamin E and increased thiobarbituric acid reacting substances (TBARs). Superoxide dismutase and other endogeneous antioxidant systems protect the blood cells from oxidative damage21.

Vitamin C acts as potent water soluble antioxidant22, 23 by scavenging physiologically relevant reactive oxygen species24, vitamin C supplementation increases glutathione in lymphocytes25, decreases single strand breaks26 and thus reduced the oxidative DNA damage27 in human lymphocytes, elevated the level of glutathione in red blood cells and improved the overall antioxidant capacity of blood28. Glutathione is an important antioxidant defense mechanism in cells which protects the cells from oxidative damage29. Recently we reported the protective effect of spirulina on phenytoin induced haematological alterations30.

The present study is proposed to evaluate the
effect of vitamin C on the hematological alterations and oxidative stress induced by phenytoin.

MATERIALS AND METHODS:

Animals

Pathogen free adult male albino Wistar rats weighing 150-200 gm were used. Male rats were chosen in order to avoid fluctuations due to oestrous cycle. The rats were inbred and housed in polypropylene cages at room temperature (25 ± 3°C) with 12/12 hours light and dark cycle and were fed with a balanced diet and tap water ad libitum. The study protocol was approved by the Institutional Animal Ethical Committee of M.S. Ramaiah College of Pharmacy, Bangalore, Karnataka. Registration No. 220/abc/CPCSEA.

Study Protocol

The rats were divided into five groups. Each group consisted of six animals.

First group served as control and received drinking water orally daily by gavage for 45 days. Second group received 20mg/Kg phenytoin dissolved in water daily by oral gavage for 45 days between 11.00 hrs and 12.00 hrs. Third, fourth and fifth groups received orally 50, 100, 200 mg/kg of ascorbic acid respectively daily for 45 days 1 hr prior to administration of (between 10.00 hrs and 11.00 hrs) 20mg/Kg p.o phenytoin.

Haematological and biochemical parameters were determined 24 hrs after the last dose. Blood samples were collected from retro orbital plexus under light ether anaesthesia. Haematological parameters include haemoglobin estimation, total erythrocyte, total leukocyte, differential leukocyte and platelet count along with packed cell volume using automated hematology analyzer XT-1800 i (Sysmex). Biochemical parameters included estimation of enzymatic antioxidants such as superoxide dismutase, catalase and non enzymatic antioxidants such as reduced glutathione, vitamin C, total antioxidant status and lipid peroxidation.

Superoxide dismutase

Assay of superoxide dismutase (SOD) was based on the ability of the enzyme to inhibit the autooxidation of pyrogallol 31. The assay was performed by taking 2.5 ml tris buffer (0.05 M) and 0.5ml EDTA (1mM) as blank. 1.5 ml tris buffer, 0.5 ml EDTA (1mM) and 1 ml pyrogallol (0.2mM) as control and the test sample consisted of 1.5 ml tris buffer (0.05M), 0.5ml EDTA (1mM), 0.05ml serum and 1ml pyrogallol (0.2mM). Change in optical absorbance per minute of sample with reference to blank was recorded at 420nm in the SICO spectrophotometer. The enzyme inhibition caused by the serum was calculated and the enzyme activity was expressed as superoxide anion reduced/mg protein/ min.

Catalase

2.5ml of phosphate buffer was added to 0.1ml of serum and incubated at 25°C for 30 min. After transferring into a cuvette the absorbance was measured at 240 nm, 650µl of hydrogen peroxide solution was added to initiate the reaction, the change in absorbance was measured for 3 min 32.

Reduced glutathione

To 0.5 ml of citrated blood, 0.5ml of 5% trichloro acetic acid (TCA) solution was added to precipitate the proteins and centrifuged at 3000rpm for 20 min. To 0.1ml of supernatant, 1ml of sodium phosphate buffer and 0.5ml of DTNB reagent was added. The absorbance of the yellow color developed was measured at 412 nm 33.

Vitamin C

To 0.5 ml of plasma, 1.5ml of 6% TCA was added and centrifuged (3500rpm/ 20min). To 0.5 ml of the supernatant. 0.5ml of DNPH reagent (2% DNPH and 4% thio urea in 9 N H2SO4) was added and developed color was read at 530nm after 30 min 34.

Total antioxidant status

0.1 ml of serum was deproteinated by the addition of 1ml of methanol, vortexed for 30 sec. It was then centrifuged at 3000rpm for 30 min to separate the proteins. To the clear supernatant 1.5 ml of methanol and 0.5ml of DPPH solutions were added, mixed thoroughly and absorbance was read at 517nm against blank. Blank was prepared in an identical way but without the addition of serum 35.

Lipid peroxidation

0.1ml of plasma was treated with 2ml of TBA 0.37%, 0.25 N HCl and 15% TCA (1:1:1 ratio) and placed in water bath for 15min, cooled and centrifuged and then clear supernatant was measured at 535nm against reference blank 36.

Statistical Analysis

The data is expressed as mean ± SEM. The data was analysed statistically using one way ANOVA, followed by Tukey Kramer test. P values less than 0.05 were considered significant.

RESULTS

Effect of chronic treatment of phenytoin and phenytoin with vitamin C on haematological profile

Table 1 summarises the effect of chronic treatment of phenytoin and phenytoin supplemented with different doses of vitamin C on Hb levels and RBC count. Chronic administration of
Phenytoin 20mg/kg for a period of 45 days significantly reduced total RBCs and haemoglobin. Vitamin C 50,100,200 mg/kg significantly increased haemoglobin while the same at a dose of 50mg/kg did not produce any significant change in total RBC count where as at higher doses i.e. 100, 200mg/kg vitamin C produced a significant increase in total RBC count as compared to chronic phenytoin treated animals.

Chronic phenytoin significantly decreased the total WBCs including neutrophils, monocytes, lymphocytes when compared with control animals. Vitamin C 50, 100, 200 mg/kg significantly reversed the phenytoin decreased WBC, neutrophils lymphocytes and monocytes (Table 2).

There is also a significant reduction in the platelets and packed cell volume in rats under chronic phenytoin treatment. Vitamin C 50,100,200

<p>| Table 1: Effect of phenytoin and phenytoin with vitamin C on RBC and haemoglobin. |
|----------------------------------------|--------------|---------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rbc</th>
<th>Haemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.78 ± 0.2595</td>
<td>16.18 ± 0.17</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>7.85 ± 0.148</td>
<td>13.33 ± 0.98</td>
</tr>
<tr>
<td>Vitamin C 50mg/kg + Phenytoin</td>
<td>7.83 ± 0.1885</td>
<td>14.25 ± 0.17</td>
</tr>
<tr>
<td>Vitamin C 100mg/kg + Phenytoin</td>
<td>8.52 ± 0.099</td>
<td>14.78 ± 0.2056</td>
</tr>
<tr>
<td>Vitamin C 200mg/kg + Phenytoin</td>
<td>8.63 ± 0.0613</td>
<td>15.68 ± 0.0744</td>
</tr>
</tbody>
</table>

Values are expressed as mean± SEM of 6 animals; *p < 0.05 vs control group; †p < 0.05 vs phenytoin group; ‡p < 0.05 vs Phenytoin + Vit C 50mg group; §p < 0.05 vs Phenytoin + Vit C 100mg; ‡p < 0.05 vs Phenytoin + Vit C 200mg.

<p>| Table 2: Effect of phenytoin and phenytoin with vitamin C on total and differential leukocytes. |
|----------------------------------------|--------------|--------------|--------------|--------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC per mm3</th>
<th>Neutrophil</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11483±124 b,c,d</td>
<td>70.4±1.43 b</td>
<td>38 ± 3.00 b,c,d</td>
<td>5.6±0.24 b,c</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>4373±480 a,c,d,e</td>
<td>53.2± 2.72 a,c,d,e</td>
<td>19±1.87 a,c,d,e</td>
<td>2.6±0.23 a,c,d,e</td>
</tr>
<tr>
<td>Vitamin C 50mg/kg + Phenytoin</td>
<td>7466±128.9 b,c,d,e</td>
<td>70.2± 2.43 b,c,d,e</td>
<td>24 ± 1.87 b,c,d,e</td>
<td>5.2±0.23 b,c</td>
</tr>
<tr>
<td>Vitamin C 100mg/kg + Phenytoin</td>
<td>8958±304.3 a,b,c,e</td>
<td>71.4± 1.43 b,c,d,e</td>
<td>35 ± 2.7 a,b,c,e</td>
<td>5.4±0.24 b,c</td>
</tr>
<tr>
<td>Vitamin C 200mg/kg + Phenytoin</td>
<td>11278±136.6 b,c,d,e</td>
<td>71.8± 3.12 b,c,e,d,e</td>
<td>38.6±2.2 b,c,d,e</td>
<td>5.4±0.24 b,c</td>
</tr>
</tbody>
</table>

Values are expressed as mean± SEM of 6 animals; *p < 0.05 vs control group; †p < 0.05 vs phenytoin group; ‡p < 0.05 vs Phenytoin + Vit C 50mg group; §p < 0.05 vs Phenytoin + Vit C 100mg; ‡p < 0.05 vs Phenytoin + Vit C 200mg.

<p>| Table 3: Effect of phenytoin and phenytoin with vitamin C on platelets and PCV |
|----------------------------------------|--------------|--------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelets</th>
<th>PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.676±0.38 a,b,c,d</td>
<td>46.5±0.39 b,c,d</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>4.64±0.93 a,b,c,d,e</td>
<td>39.16±0.31 a,b,c,d</td>
</tr>
<tr>
<td>Vitamin C 50mg/kg + Phenytoin</td>
<td>7.59±0.17 a,b,c,d,e</td>
<td>40.98±0.59 a,b,c,d,e</td>
</tr>
<tr>
<td>Vitamin C 100mg/kg + Phenytoin</td>
<td>8.5±0.26 a,b,c,e</td>
<td>42.96±0.38 a,b,c,e</td>
</tr>
<tr>
<td>Vitamin C 200mg/kg + Phenytoin</td>
<td>8.676±0.16 b,c,d</td>
<td>44.96±0.35 b,c,d</td>
</tr>
</tbody>
</table>

Values are expressed as mean± SEM of 6 animals; *p < 0.05 vs control group; †p < 0.05 vs phenytoin group; ‡p < 0.05 vs Phenytoin + Vit C 50mg group; §p < 0.05 vs Phenytoin + Vit C 100mg; ‡p < 0.05 vs Phenytoin + Vit C 200mg.

<p>| Table 4: Effect of chronic treatment of phenytoin and phenytoin with vitamin C on superoxide dismutase, catalase and lipid peroxidation |
|----------------------------------------|--------------|--------------|--------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>SOD Superoxide anion reduced / mgprotein / min</th>
<th>Catalase µmol H2O2 degraded / mgprotein / min</th>
<th>Lipid peroxidation mmol/dl/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.83±0.2985 a,b,c,d</td>
<td>59.86±0.203 b,c,e</td>
<td>4.65±0.26 b,c,d,e</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>2.113±0.177 a,b,c,d,e</td>
<td>40.198 ± 0.32 a,b,c,d,e</td>
<td>9.7±0.17 a,b,c,d,e</td>
</tr>
<tr>
<td>Phenytoin + Vit C 50mg</td>
<td>2.665±0.087 a,b,c,d</td>
<td>42.645 ± 0.06 a,b,c,d</td>
<td>9.85±0.17 a,b,c,d,e</td>
</tr>
<tr>
<td>Phenytoin+Vit C 100mg</td>
<td>3.145±0.127 a,b,c</td>
<td>44.18 ± 0.12 a,b,c,d,e</td>
<td>9.0±0.17 a,b,c,d,e</td>
</tr>
<tr>
<td>Phenytoin+Vit C 200mg</td>
<td>3.498±0.077 a,b,c</td>
<td>45.42 ± 0.20 a,b,c,d,e</td>
<td>8.57±0.13 a,b,c,d,e</td>
</tr>
</tbody>
</table>

Values are expressed as mean± SEM of 6 animals; *p < 0.05 vs control group; †p < 0.05 vs phenytoin group; ‡p < 0.05 vs Phenytoin + Vit C 50mg group; §p < 0.05 vs Phenytoin + Vit C 100mg; ‡p < 0.05 vs Phenytoin + Vit C 200mg.
mg/kg significantly increased platelets and packed cell volume when compared to chronic phenytoin treated rats (Table 3).

**Effect of chronic treatment of phenytoin and phenytoin with vitamin C on endogenous antioxidants, total antioxidant status and lipid peroxidation.**

Chronic phenytoin treatment significantly decreased the superoxide dismutase levels when compared to control animals. Vitamin C at the dose of 50mg/kg did not reverse the phenytoin reduced superoxide dismutase levels, whereas 100 and 200mg/kg of Vitamin C significantly increased the superoxide dismutase levels when compared to phenytoin treated animals. Chronic phenytoin treatment significantly decreased the catalase levels when compared to control animals. Vitamin C at the dose of 50mg/kg did not reverse the phenytoin reduced catalase, whereas 100 and 200mg/kg of Vitamin C significantly increased the catalase levels when compared to phenytoin treated animals (Table 4).

Table 5 shows the effect of chronic treatment of phenytoin, phenytoin + vitamin C on reduced glutathione and vitamin C levels. Chronic phenytoin treatment significantly decreased the reduced glutathione levels when compared to control animals. Vitamin C at the dose of 50mg/kg did not reverse the phenytoin depleted reduced glutathione levels, whereas 100 and 200mg/kg of Vitamin C significantly increased the reduced glutathione levels when compared to phenytoin treated animals. Chronic phenytoin treatment significantly decreased Vitamin C levels when compared to control animals. Vitamin C at the dose of 50, 100 and 200mg/kg significantly increased Vitamin C levels when compared to phenytoin treated animals.

Chronic phenytoin treatment significantly decreased the total antioxidant levels when compared to control animals. Vitamin C at the dose of 50mg/kg did not reverse the reduced total antioxidant levels, whereas 100 and 200mg/kg of Vitamin C significantly increased the phenytoin decreased total antioxidant levels. Vitamin C treatment at the dose of 100 mg/kg increased the plasma vitamin C level almost very close to the control and 200mg/kg elevated the plasma vitamin C levels more than the control (Table 5).

Chronic phenytoin treatment significantly increased lipid peroxidation when compared to control animals. Vitamin C at the dose of 50mg/kg did not reverse the lipid peroxidation, whereas 100 and 200mg/kg of Vitamin C significantly decreased the phenytoin increased lipid peroxidation (Table 4).

**DISCUSSION**

In the present study chronic treatment with phenytoin caused a significant decrease in total erythrocytes, total leucocytes, neutrophils, monocytes, lymphocytes and platelets. Vitamin C 50, 100 and 200mg/kg for 45 days significantly increased the phenytoin reduced blood cells, haemoglobin content and packed cell volume. Phenytoin decreased enzymatic antioxidants such as SOD, catalase and non enzymatic antioxidants like reduced GSH, Vitamin C, also decreased total antioxidant status and increased lipid peroxidation. Vitamin C 100 and 200mg/kg for 45 days significantly augmented the phenytoin reduced enzymatic, non enzymatic antioxidants, total antioxidant status and decreased the lipid peroxidation.

**Effect of phenytoin on haematological parameters**

Phenytoin undergoes oxidative metabolism with the formation of potentially toxic arene oxide intermediates 37, which binds covalently to cell macromolecules and cause cytotoxic damage and bone marrow toxicity 40, aplastic anemia. The drug is shown to suppress mitogen induced activation of lymphocytes 41, 42, T lymphocyte activity 42, 43, 44, 45, 46 and thereby depresses immunological function47, 48, 49, 50. Hematological abnormalities

### Table 5: Effect of chronic treatment of phenytoin and phenytoin with vitamin C on reduced glutathione, vitamin C and total antioxidant status.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (mg/ dl)</th>
<th>Vit C (mg/ dl)</th>
<th>TAS (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.94±0.19</td>
<td>17.4±0.994</td>
<td>0.944±0.125</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>11.48±1.6</td>
<td>7.64±0.23</td>
<td>0.568±0.011</td>
</tr>
<tr>
<td>Phenytoin+ Vit C 50mg</td>
<td>11.89±21</td>
<td>12.56±465</td>
<td>0.572±0.007</td>
</tr>
<tr>
<td>Phenytoin+ Vit C 100mg</td>
<td>12.77±21</td>
<td>17.25±0.59</td>
<td>0.605±0.016</td>
</tr>
<tr>
<td>Phenytoin+ Vit C 200mg</td>
<td>14.54±14</td>
<td>19.15±895</td>
<td>0.67 ± 0.006</td>
</tr>
</tbody>
</table>

Values are expressed as means SEM of 6 animals; * p < 0.05 vs control group; ** p < 0.05 vs phenytoin group; *** p < 0.05 vs Phenytoin + Vit C 50mg group; p < 0.05 vs Phenytoin + Vit C 100mg; * p < 0.05 vs Phenytoin + Vit C 200mg
induced by phenytoin include leucocytosis with atypical lymphocytes, eosinophilia and agranulocytosis. An immune mechanism with phenytoin dependent antigranulocyte antibody causes leucopenia which resolves on discontinuing the therapy. Phenytoin has a direct toxic effect and results in pancytopenia and agranulocytosis. Tressly reduces packed cell volume as well. Thrombocytopenia is evident within 15 days of initiation of phenytoin therapy. Online with this in the present study also phenytoin decreased the erythrocytes, total leucocytes, monocytes, lymphocytes, neutrophils and thrombocytes. In our previous study we reported that the supplementation of spirulina has reversed the haematological disturbances induced by phenytoin and carbamazepine. In the present study also we observed that vitamin C has augmented chronic phenytoin therapy induced alterations in RBC, WBC, platelet counts, hemoglobin and packed cell volume.

**Effect of phenytoin on oxidative stress**

Oxidative stress is the shift in balance in cellular oxidation – reduction reactions in favour of oxidation, leading to cellular damage and is indicated by oxidized products of lipids, proteins and nucleic acids.

Phenytoin is bioactivated to reactive intermediates that bound irreversibly to macromolecules in neutrophils. Phenytoin caused abnormal metabolism of super oxide anion reduced 40% of glutathione (GSH) content. Glutathione is an important antioxidant defense mechanism in living cells. Decreased tissue glutathione concentrations are associated with cell damage, depressed immunity. Phenytoin and its intermediates are proved to produce free radicals and thereby elevate the lipid peroxidation and reduce the antioxidants like glutathione, catalase, superoxide dismutase and total antioxidant capacity. In the present study phenytoin decreased the enzymatic and non enzymatic antioxidants such as SOD, catalase, reduced GSH, vitamin C, also total antioxidant status and increased lipid peroxidation.

**Effect of vitamin C on oxidative stress and haematological parameters**

In the present study vitamin C reversed the phenytoin decreased GSH concentration. Previous study also reported the ameliorative effect of vitamin C on reduced glutathione concentration in blood, red blood cells and improved the overall antioxidant protection capacity of blood. It produced reduction in plasma GSSG (oxidized glutathione), which shows a better index of antioxidant status and oxidant protection. Vitamin C supplementation increased the glutathione, SOD and catalase activities in lymphocyte. Thus vitamin C is proposed to reduce oxidative stress from H₂O₂ potentially by reducing the free radical species generated from H₂O₂. Vitamin C reduces oxidative DNA damage also decreased single strand breaks in human lymphocytes. Vitamin C supplementation also reduced 8-oxo deoxyguanosine (oxidized DNA) in white blood cells. In the present study vitamin C reversed phenytoin induced decrease in GSH, Vit C, SOD, catalase, total anti oxidant status and increase in lipid peroxidation thereby protected the erythrocytes, leucocytes and platelets from oxidative stress.

The results of the present study indicated phenytoin treatment decreased the levels of SOD, CAT, GSH, Vitamin C, and TAS whereas it increased the lipid peroxidation, also caused a decrease in RBCs, WBCs, platelets, haemoglobin and packed cell volume. Vitamin C improved the serum enzymatic and non enzymatic antioxidant levels, as well as increased the total antioxidant capacity whereby decreased the lipid peroxidation, also increased blood cell count, haemoglobin and packed cell volume.

The present results support the view that vitamin C (100 and 200mg/Kg) protects against phenytoin induced hematotoxicity and oxidative stress in rats. The effects of ascorbic acid are believed to be related to its intrinsic ability to scavenge free radicals. This investigation reports the curative effect of vitamin C on phenytoin induced toxicity. However further work on effectiveness of vitamin C supplementation in epileptic patients is required.

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**REFERENCES**


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