CHAPTER IV
EXPERIMENTAL FINDINGS
CHAPTER IV

EXPERIMENTAL FINDINGS

General Studies

General health status of the treated animals was deteriorated gradually following long term caffeine consumption that was revealed by the loss of body weight and other behavioral changes. During experimental period animals were kept under constant supervision and care. Behavioral alterations were appeared to be due to psychotropic nature of the chemicals, which was expressed as loss of body weight, apathy to food intake, nervousness, hypersensitivity etc.

Loss of body weight following chronic caffeine administration reflected the toxicity of chemical compound and considered as a prime area in toxicological research. A gradual loss of body weight appeared after caffeine administration in caffeine fed rat and also caffeine and vitamin-C suplimented rat but the control rat showed a normal level.

As recorded there was a fluctuation of body weight in some of the rats, however the overall loss of body weight was confirmed.
A decline of body weight was observed in both the treated groups than control at the early part of the experiment but at later body weight gained which indicated the development of tolerance against caffeine toxicity.

Rats were allowed to take food sufficiently with standard laboratory diet. Besides some nutritional supplements also given at regular intervals in all the groups of rat.

During the experimental observation it was found that animals refused to take food after administration of the toxicants and water intake enhanced in caffeine-administered group. Simultaneously ascorbic acid supplemented rat along with caffeine also showed a refusal tendency for food but enhanced water intake. Such behavioral change was observed throughout the experimental period and rats of both the treated groups generally taken food after 6 hours of the administration of the toxicants.

Recognizable morphological changes were not observed during the experimental period but a markable change of behavior was observed. Especially after 2 hours of caffeine treatment, treated rat showed an aggressive behavior, and also became lethargic.

Abnormalities in behavioral pattern was observed in the experiment. Immediately after administration of the toxicant rats of both the treated groups showed hyper motility such as tail tremor, jerking movement and also jaw movement. This
abnormality persists for about 2 hours after the administration of the toxicant. Even after the 5 hours they also showed highly sensitive to touch and sound and also with aggressiveness. After 5 hours of the dose, rats became lethargic and sluggish and also showed the loss of activity. Animal spented more time lying immobile, stretching on the cage bottom showing a morbid effect during the day. The hyperactivity persisted only for 5 hours.

Continuation of the exposure, rats became able to tolerate about the toxicity of caffeine and some abnormalities like hyper motility, and sensitivity became declined. Others behavioral alteration remained as such through out the experimental period.

The respiration or respiratory rate also changed after caffeine administration. Rate of respiration became quicken after the dose for at least 5 hours and than became normal. Rats also showed spasmodic movement of limb.

Caffeine level in blood

Caffeine, purine derivative is a not a normal constituent of mammalian system appear in the blood tissues and organs after ingestion only. Distribution of caffeine on various organs and tissues varies, but it is almost evenly distributed in the body fluid. A considerable amount of caffeine accumulates in various organs of the body after repeated and prolong intake. However, available literature does not reveal any information regarding the quantity of day to day accumulation of caffeine in the
tissues or organs. Absorption of caffeine occurs vary rapidly and completes after one hour from the gastrointestinal tract following ingestion.

In the present investigation the quantitative estimation of caffeine in 0 day blood samples i.e the sample collected prior to caffeine feeding showed no trace of caffeine (Table-1). This finding was observed in all the three groups of rats before administration of caffeine and caffeine plus vitamin C. As the control group of rats were reared under caffeine free diet, therefore in subsequent period of the experiment also caffeine could not be traced in their blood.

In the caffeine treated group, after 10 days of caffeine administration, there was significant (p< 0.01) rise of caffeine level in blood as compared to the controls. This level was also significantly higher than that of the animal group that received caffeine and vitamin C as antitoxic compound. However, the serum caffeine concentration on day of caffeine administration did not show significant difference in its level as compared to the level in the caffeine and vitamin C supplemented rats. Conversely, the caffeine fed rats showed significantly (p<0.01) higher level of caffeine on day 30 following caffeine administration as compared to the rats, which received vitamin C supplement in addition to caffeine. As showed in Table-1, the level of caffeine in the serum of caffeine plus vitamin C treated rats on day 40 of treatment were estimated to be 154.23 ± 4.67 and 159.17 ± 2.32 microgram/ml respectively.
In the present study, the statistical analysis revealed non-significant differences of caffeine level in the blood between the caffeine and caffeine plus vitamin C treated group.

Perusal to table-1 with the advancement of treatment period significant differences of serum caffeine level were observed between the treated groups. On the day 50 and 60 of treatment, the serum caffeine concentrations were significantly (p<0.01) higher in caffeine plus vitamin C treated group than that of only caffeine treated one. On the subsequent period of the treatment i.e. on day 70, 80 and 90 the serum caffeine concentration declined in caffeine plus vitamin C treated group as compared to day 60 of treatment and the values were recorded to be 223.95, 204.46 and 205.01 microgram/ml respectively. These levels of serum caffeine were found to be significantly (p<0.01) higher than those of only caffeine treated group.

Perusal to figure-1 revealed a steep rise of serum caffeine concentration on day 30 of caffeine treatment as compared to 10 and 20 days. A rise of serum caffeine level was also observed on day 50 of caffeine plus vitamin C treated group. The statistical analysis also revealed this value to be significantly higher than those of values recorded following short and very long period of treatment.
Table 1
Average concentration of caffeine (microgram/ml) in blood in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SE</td>
<td>CV%±SE</td>
<td>Mean ±SE</td>
<td>CV%±SE</td>
<td>Mean ±SE</td>
<td>CV%±SE</td>
<td>Mean ±SE</td>
<td>CV%±SE</td>
<td>Mean ±SE</td>
<td>CV%±SE</td>
</tr>
<tr>
<td>Group I</td>
<td>NIL</td>
<td>0</td>
<td>NIL</td>
<td>0</td>
<td>NIL</td>
<td>0</td>
<td>NIL</td>
<td>0</td>
<td>NIL</td>
<td>0</td>
</tr>
<tr>
<td>Group II</td>
<td>NIL</td>
<td>0</td>
<td>113.82 ±73</td>
<td>1.57</td>
<td>115.96 ±29</td>
<td>2.73</td>
<td>159.17 ±4.02</td>
<td>6.19</td>
<td>154.23 ±467</td>
<td>1.64</td>
</tr>
<tr>
<td>Group III</td>
<td>NIL</td>
<td>0</td>
<td>106.39 ±2.29</td>
<td>5.29</td>
<td>111.01 ±401</td>
<td>8.86</td>
<td>152.03 ±0.89</td>
<td>1.66</td>
<td>159.17 ±232</td>
<td>0.86</td>
</tr>
</tbody>
</table>

N.B. - Sub class means in column with different superscripts differ significantly (P<0.05). Within parameter are the coefficient of variance %

NS - Values are non significant at P<0.05 level.
Figure 1. Line diagram showing the level of Caffeine in blood

Figure 1. Bar diagram showing the level of Caffeine in blood
Vitamin C level in blood

The antitoxic effect of vitamin C has led to its meaningful use to reduce toxic effect of harmful substances. Although it is widely distributed in the body, its concentration varied in different organs and tissues as parallel to their metabolic need. As estimated, maximum of its concentration in the body remains in the reduced form with a relatively small amount as dehydroascorbic acid. Lower animal like rat can synthesize vitamin C endogenously from glucose by uronic acid pathway.

In the present investigation, vitamin C was estimated prior to and following caffeine and vitamin C treatment at various time interval. Prior to the treatment, the concentration of vitamin C in blood was found to be almost identical in all the group of rats. The levels of vitamin C in the serum of group I, II and III animals were recorded as 0.85 ± 0.04, 0.87 ± and .94 mg%, respectively (Table 2). The control rats which were reared under caffeine free diet and without vitamin C supplement showed normal levels of vitamin C throughout the experimental period.

Following the treatment of caffeine and caffeine plus vitamin C, the concentration of vit.c in blood showed significant (p .01) variation in both groups and also between periods. Perusal to table 2, 30 days exposure caffeine resulted in peak value (1.27 ±0.07 mgo/0) of vitamin C, which was significantly higher than those of
control values. A similar rise of vitamin C was also observed on day 30 in the caffeine plus vitamin C treated rats. The peak level of vitamin C \( (1.96 \pm .19 \text{ mg\%}) \) however, was recorded in rats on day 90 which received the treatment of both caffeine and vitamin C. This concentration of serum vitamin C was found to be significantly higher as compared to the same days exposure values i.e. of day 90 in caffeine fed rats without vitamin C supplementation.

Perusal to figure 2, the level of vitamin C in blood showed a rise following 30 days caffeine and caffeine plus vitamin C treatment and such high levels of vitamin C were almost maintained throughout the rest of the experimental period. Statistical analysis also revealed significantly \( (p < .01) \) higher concentration of vitamin C in blood following different period of caffeine and caffeine plus vitamin C treatment as compared to control group of rats (Table 2).
Table 2
Average concentration of Vitamin C (mg/dl) level in blood in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Group I</td>
<td>0.8513 ± 0.04</td>
</tr>
<tr>
<td>Group II</td>
<td>0.8704 ± 0.03</td>
</tr>
<tr>
<td>Group III</td>
<td>0.9399 ± 0.03</td>
</tr>
</tbody>
</table>

N.B.: Sub class means in column. Within parameter are the coefficient of variance %
NS Values are non significant at P<0.05 level.
Figure 2. Line diagram showing the level of vitamin C in blood

Figure 2. Bar diagram showing the level of vitamin C in blood
Glucose level in blood

Toxicological impact on the physiological processes in the body often reflected upon the blood glucose level and therefore it has got primary importance to determine the blood glucose levels in toxicological research. Glucose level in blood is regulated by several factors in a chain like manner and any disturbing agent can alter the blood glucose to abnormal status. The present investigation is also designed to evaluate the actual scenario of the impact of caffeine on carbohydrate metabolism and most relevant blood parameter the insulin.

In the present experiment no significant variation was observed in respect of blood glucose following fasting in all the experimental animal group prior to administration of caffeine and vitamin C. Further the table 3 showed that the glucose level in the blood increased significantly (P < .0 1) following 10 days of caffeine and caffeine plus vitamin C treatment. However, there was no significant difference in blood glucose levels between the treatment groups after 20 days exposure to caffeine only and caffeine plus vitamin C. The peak concentrations of blood sugar (108.01± 2.07) and 108.73 ± 2.86 mg%) in both the treated groups after the same days (20 days) of treatment with significantly less control value (70.41 ± 4.56 mg%).

The present experiment also showed that after 30 days of caffeine administration the level of glucose in-group H declined and became significantly lower
than the value recorded for group III. However, the blood glucose concentrations of both the caffeine and caffeine plus vitamin C treated rats showed significantly ($p < 0.01$) than that of control one. Perusal to table 3, there was significant elevation of blood glucose level up to 40 days of treatment in group III, and thereafter it declined to the control level, while the group III rats showed significantly higher values as compared to the caffeine treated and the control group.

Further the present study showed that, following 50 days of treatment and thereafter the blood glucose concentration declined to control level as statistical analysis failed to show any significant differences of blood glucose between the treated and control group.
Table 3
Average concentration of blood glucose (mg/dl) in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Group I</td>
<td>6781±209</td>
</tr>
<tr>
<td>Group II</td>
<td>7471±369</td>
</tr>
<tr>
<td>Group III</td>
<td>6892±148</td>
</tr>
</tbody>
</table>

N.B. – Sub class means in column with different superscripts differ significantly (P<0.05). Within parameter are the coefficient of variance %
NS Values are non significant at P<0.05 level.
Figure 3. Line diagram showing the level of Glucose in blood

Figure 3. Bar diagram showing the level of Glucose in blood
Insulin level in blood

Insulin plays vital role in carbohydrate, protein and lipid metabolism. More pronouncedly, it is associated with the change of glucose level in blood. Hence, the insulin essentially represents body’s defense mechanism as any alteration in the level of insulin may cause malfunctioning of the organs or life. In case of risk assessment arising out of caffeine consumption, determination of insulin in blood is of major importance.

In the present study the blood insulin levels were estimated following RIA technique in the blood samples collected on fasting and at different time interval in caffeine and caffeine plus vitamin C treated experimental rats to study the effect of short and long term feeding of caffeine on this pancreatic hormone and antitoxic effect of vitamin C.

As showed in table 4, the blood insulin level did not differ significantly between different groups of animals prior to the treatment of caffeine and vitamin C. However, as observed after 10 days of treatment the blood insulin level showed significantly higher values with a peak value (69.50 ± 6.72 uU/ml) on day 90 of caffeine plus vitamin C treatment against the control value of only 33.00 ± 2.52 uU/ml (Figure 4). In only caffeine fed rats the peak value of insulin in blood was recorded as 51.33 ± 2.23 uU/ml following 80 days of treatment and was significantly higher than that of control rats. Perusal to figure-4, an elevation of blood insulin was observed on day 10 of caffeine and caffeine plus vitamin C treated rats which was thereafter maintained at
higher levels till the end of the experimental period i.e. till 90 days. Long term treatment of caffeine plus vitamin C (day 70, 80 and 90) as observed in group III, caused significant rise of insulin level in blood as compared to only caffeine treated one.
Table 4
Average serum concentration of insulin (uU/ml) in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean ±SE</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32.00 NS</td>
</tr>
<tr>
<td></td>
<td>± 3.00</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.16 NS</td>
</tr>
<tr>
<td></td>
<td>± 0.06</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.50 NS</td>
</tr>
<tr>
<td></td>
<td>± 0.03</td>
</tr>
</tbody>
</table>

N.B. - Sub class means in column with different superscripts differ significantly (P<0.05). Within parameter are the coefficient of variance %. NS Values are non significant at P<0.05 level.
Figure 4. Line diagram showing the level of Insulin in blood

Figure 4. Bar diagram showing the level of Insulin in blood
Protein level in blood

Proteins are very complex mixture of proteins, changes of which may result in many pathological condition. Dietary influence on plasma protein level is an area of toxicological importance became significance in maintaining homeostasis of the body.

In the present investigation it was observed that the serum protein level did not differ significantly prior to caffeine and vitamin C treatment. As shown in the table 5 the 0 day level were recorded as 7.53, 7.38 and 7.00 gm percent in groups I, II and III respectively. As observed on 30, 60 and 90 days of caffeine and caffeine plus vitamin C treatment, there was no significance difference of serum protein levels between non-treated and caffeine and caffeine plus vitamin C treated sets till 60 days of treatment. However, the protein levels shows a significance (P<.01) decrease on day 90 in both the caffeine and caffeine plus vitamin C treated groups as compared to the control values. The serum protein level on day 90 in group I, II and III were recorded as 7.6, 7.03 and 6.66 gm percent respectively. Perusal to figure 5 revealed a declined level of serum protein in day 90 following a rise on day 60 on the treatment in both the treated groups.
Table 5
Average concentration of blood protein (mg/dl) in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
<th>Mean + SE</th>
<th>CV%</th>
<th>Mean + SE</th>
<th>CV%</th>
<th>Mean + SE</th>
<th>CV%</th>
<th>Mean + SE</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>60</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.B.: Sub class means in column. Within parameter are the coefficient of variance %. NS values are non significant at P<0.05 level.
Figure 5. Line diagram showing the level of Protein in blood

Figure 5. Bar diagram showing the level of Protein in blood
Uric Acid level in blood

Uric acid is an end product of nitrogen metabolism. The compound itself and its salts were highly insoluble and toxic and thus increase of its level causes clinical problems. As the mammalian system has less tolerance to this compound, accordingly they had the capability to convert it to a highly soluble non toxic compound urea. Hence quantitative estimation of uric acid and urea is of scientific importance in relation to nitrogen metabolism in the assessment of uric acid level.

In the present Investigation, the uric acid concentration in the blood of group I, II and III rats were recorded as 2.36, 2.48, and 2.55 mg percent respectively before commencement of caffeine and caffeine plus vitamin C treatment. An apparent decrease of uric acid level was detected on day 30 of caffeine administration (group II) while its showed an elevated level on day 60 of caffeine treatment. Perusal to figure 6 also indicated a decline of uric acid level on day 60. However, in group III rats which received both caffeine and vitamin C treatment showed an increase in trend of uric acid level beginning on day 30 through days 60 and 90 of the treatment. Average of data showed that there was no significant difference of uric acid level in blood between treated and non treated groups.
Table 6
Average concentration of blood uric acid level (mg/dl) in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>30</td>
<td>60</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (±SE)</td>
<td>CV%</td>
<td>Mean (±SE)</td>
<td>CV%</td>
<td>Mean (±SE)</td>
<td>CV%</td>
<td>Mean (±SE)</td>
</tr>
<tr>
<td>Group I</td>
<td>2.36 NS ± 0.05</td>
<td>5.41</td>
<td>2.57 NS ± 0.03</td>
<td>8.42</td>
<td>2.58 NS ± 0.06</td>
<td>5.74</td>
<td>2.42 NS ± 0.09</td>
</tr>
<tr>
<td>Group II</td>
<td>2.48 NS ± 0.08</td>
<td>8.14</td>
<td>2.08 NS ± 0.13</td>
<td>15.78</td>
<td>2.74 NS ± 0.28</td>
<td>25.66</td>
<td>2.37 NS ± 0.16</td>
</tr>
<tr>
<td>Group III</td>
<td>2.55 NS ± 0.08</td>
<td>7.97</td>
<td>2.29 NS ± 0.27</td>
<td>29.02</td>
<td>2.64 NS ± 0.21</td>
<td>19.78</td>
<td>2.69 ± 0.20</td>
</tr>
</tbody>
</table>

N.B. : Sub class means in column. Within parameter are the coefficient of variance %.
NS Values are non significant at P<0.05 level.
Figure 6. Line diagram showing the level of Uric acid in blood

Figure 6. Bar diagram showing the level of Uric acid in blood
Urea level in blood

Level of urea were quantified in all the rats of different groups before treatment and no statistically significant variations were found. Urea levels were estimated in all the groups at 10 days intervals and after 12 hours of caffeine administration. During chronic treatment concentration of urea that were found before administration of drugs were considered as 0 day value and other values were considered as value on day of sample collection. Although a little variation were found in different groups on 0 day level but were non significant statistically.

Result of experiment showed that on 0 day of chronic therapy, urea level were not varied significantly among different groups .An indication of significant variations appeared after 20 days of chronic treatment where urea concentration enhanced in group II and group III than group I and it was found that more urea were detected in the blood of group II and group III (table 7)

A recognizable elevation of blood urea appeared after 40 day of caffeine administration with highly significant values. Group II had high urea level than group III

Group II again showed high value than group III on day 50 and group I had comparatively low level than both the treated groups . On the day 60 , blood urea level were low in group II than group III but not with group I.
At the later part of the experiment, results were apparently similar and observed a high value on group II than III on day 70, 80 and 90 or upto the end of the experiment. It was notable that level of urea in control group or group I showed lower level than caffeine administered or group II and caffeine with ascorbic acid treated group or group III.

On comparison with 0 day level of urea of same group, no statistically significant variation were detected on control group of animal at different day.

In caffeine administered group level of urea were compared with 0 day level of same group and found that level remained same after 10 day of caffeine administration and detectable change occurred after 20 day of caffeine treatment and became highly significant. Although level of urea fluctuating as found on the collected samples of different day but peak elevated level appeared on day 40 and than became slightly decline with significant higher than that control.

In the caffeine and ascorbic acid administered group level of urea remained almost same upto the 10th day with 0 day level and on day 20 level found to be higher. A highly elevated urea level was found on 40 and 50 day of treatment. On day 60 level of urea declined and remained higher than 0 day level and also showed high value upto the end of the experiment.
Table 7
Average concentration of blood urea (mg/dl) in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ±SE</td>
<td>CV%</td>
<td>Mean ±SE</td>
<td>CV%</td>
<td>Mean ±SE</td>
<td>CV%</td>
<td>Mean ±SE</td>
<td>CV%</td>
<td>Mean ±SE</td>
<td>CV%</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
<td>30.72 ±2.13</td>
<td>17.02</td>
<td>30.79 ±1.65</td>
<td>13.17</td>
<td>31.13 ±1.65</td>
<td>13.04</td>
<td>32.03 ±1.18</td>
<td>9.02</td>
<td>29.98 ±1.13</td>
<td>9.28</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td>29.43 ±1.17</td>
<td>9.81</td>
<td>27.59 ±1.03</td>
<td>9.34</td>
<td>45.51 ±2.20</td>
<td>11.86</td>
<td>34.90 ±0.95</td>
<td>6.71</td>
<td>52.93 ±4.89</td>
<td>25.44</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td>28.06 ±0.66</td>
<td>5.77</td>
<td>27.33 ±2.19</td>
<td>19.63</td>
<td>35.72 ±1.82</td>
<td>12.51</td>
<td>42.22 ±3.90</td>
<td>22.64</td>
<td>48.14 ±4.70</td>
<td>23.91</td>
</tr>
</tbody>
</table>

N.B. - Sub class means in column with different superscripts differ significantly (P<0.05). Within parameter are the coefficient of variance %
NS Values are non significant at P<0.05 level.
Figure 7. Line diagram showing the level of Urea in blood.

Figure 7. Bar diagram showing the level of Urea in blood.
Cholesterol level in blood

Cholesterol is an amphipathic lipid and is present in all tissues and plasma, forming an essential components of the membrane structure of the cell. Several factors like fasting, certain food components including fat level in diets affect endogenous production of cholesterol. Caffeine, which is habitually consume compound may exerts influence on metabolic pathways to alter the plasma cholesterol level and thus invites toxicological importance in relation to cardiovascular system.

In the present investigation, serum cholesterol level were estimated to be 51.00, 48.50 and 56.06 mg percent in group I, II, and III respectively prior to caffeine and caffeine plus vitamin C treatment (Table 8). Statistical analysis of the estimated data did not show significance difference of the serum cholesterol level between non treated and treated groups (Table 8). However, serum cholesterol level declined from 30 days of treatment through 60 and 90 days in both the caffeine and caffeine plus vitamin C treated rats. Lower level of serum cholesterol were also observed on day 60 and 90 in both the groups as compared to control values.
Table 8
Average concentration of blood cholesterol (mg/dl) in different groups, along with results of C.D. Test at different days of treatment. Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean±SE</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51.00&lt;sup&gt;NS&lt;/sup&gt;± 2.75</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48.50&lt;sup&gt;NS&lt;/sup&gt;± 2.41</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56.06&lt;sup&gt;NS&lt;/sup&gt;± 4.61</td>
</tr>
</tbody>
</table>

N B. : Sub class means in column. Within parameter are the coefficient of variance %.<sup>NS</sup> Values are non significant at P<0.05 level.
Figure 8. Line diagram showing the level of Cholesterol in blood

Figure 8. Bar diagram showing the level of Cholesterol in blood
Determination of thyroid profiles

Triiodothyronine and tetraiodothyronine occupy an important position in hormone physiology and these hormones regulates basal metabolic rates. Besides diet several other factors are involved in the synthesis of the thyroid hormone T₃ and T₄ and their maintenance in the blood. A complex mechanism is evolved to acquire and retain the dietary elements iodine to form this hormones in the thyroid follicles. Hence, the study of injurious affects of some dietary components on the level of these hormones in blood is considered important from the risk assessment point of view.

Triiodothyronine

In the present investigation T₃ assayed before and after treatment of caffeine and caffeine plus vitamin C in experimental rats. As shown in the table 9 0 day level of serum T₃ levels were estimated to be $0.80 \pm 0.05, 0.66 \pm 0.06$ and $0.76 \pm 0.03$ ng per ml in group I, II and III respectively. Following administration of the test compounds and as estimated on day 10 the serum T₃ levels were found to be significantly lower (P<0.01) in both the treated groups (II and III) then that of control values ($0.90 \pm 0.08$ ng per ml).
As the administration of caffeine and caffeine plus vitamin C continued, the serum T₃ level were also continued to be significantly lowered as estimated on day 20, 30, 40, 50, 60, 70, 80 and 90 as compared to their control values.

Perusal to the figure 9 also indicated that serum T₃ level on various days of treatment were maintained at almost identical lower values through the experimental period in caffeine with vitamin C supplemented rats. The group II of rats which received only caffeine treatment without vitamin C supplements showed a peak value of serum T₃ (0.64 ± 0.04 ng per ml) on day 60. However, this peak value was comparable to the mean value of serum T₃ as estimated in the same group of animals on 0 day.
Table 9
Average serum concentration of Triiodothyronine (ng/ml) in different groups, along with results of C.D. Test at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Group I</td>
<td>0.80 NS ± 0.05</td>
</tr>
<tr>
<td>Group II</td>
<td>0.66 NS ± 0.06</td>
</tr>
<tr>
<td>Group III</td>
<td>0.76 NS ± 0.03</td>
</tr>
</tbody>
</table>

N.B. – Sub class means in column with different superscripts differ significantly (P<0.05). Within parameter are the coefficient of variance %
NS Values are non significant at P<0.05 level.
Figure 9. Line diagram showing the level of Serum concentration of Triiodothyronine in blood

Figure 9. Bar diagram showing the level of Serum concentration of Triiodothyronine in blood
Tetraiodothyronine level

The T4 concentration in blood on day 0 in group I, II and III were recorded as 92.00 ± 4.52, 95.50 ± 3.89 and 85.00 ± 4.12 respectively (Table 10). Following the administration of caffeine and caffeine plus vitamin C (group III) the T4 concentration in the blood varied significantly (P<0.05) between treated and non treated rats at various days. In caffeine fed rats, the level of serum T4 were significantly lower on 10, 20, 30, 40, 50 and 60 days of treatment. A similar and significantly lower values of the T4 were also observed in group II on identical days as in group III. However, both the treated groups showed non significant in their serum T4 levels beyond 60 days being observed on 70, 80 and 90 days. Statistical analysis of the estimated data between treatment groups revealed that the levels of serum of T4 were significantly higher in group III on day 50 and 60 as compared to the values of group II rats.

Conversely group II rats showed significantly higher value of serum T4 on day 20 and 30 as compared to group III rats. Perusal to the figure-10 failed to show any definite trend of T4 concentration with the period of treatment of caffeine and caffeine plus vitamin C.
Table 10
Average serum concentration of tetradiodothyronine (ng/ml) in different groups, along with results of C.D. Test at different days of treatment. 
Data were compared between group at different days of treatment.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Days of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
</tr>
<tr>
<td>0.00 NS ± 4.52</td>
<td>12.05</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
</tr>
<tr>
<td>9.50 NS ± 3.89</td>
<td>9.99</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
</tr>
<tr>
<td>8.50 NS ± 4.12</td>
<td>11.88</td>
</tr>
</tbody>
</table>

N.B. – Sub class means in column with different superscripts differ significantly (P<0.05). Within parameter are the coefficient of variance %
NS Values are non significant at P<0.05 level.
Figure 10. Line diagram showing the level serum concentration of Tetraiodothyronine

Figure 10. Bar diagram showing the level serum concentration of Tetraiodothyronine
Effect of caffeine on erythrocytes:

Results of the experiment showed that caffeine appeared to cause deformation of erythrocytes resulting in the formation of different types of abnormally shaped cells. In the early part, transformation of discocytes to echinocytes was evident in caffeine treated animals, although well-defined echinocytes were insignificant in numbers.

Membrane internalization and wrinkling of red blood corpuscles were also seen in caffeine administered rat. This type of membrane deformation was although found, but their numbers were quite low. Erythrocytes modification of this type included uniform investigations of membranes and larger longitudinal infoldings of the membranes leading to shrinkage of the cells.

Another deformed erythrocyte formed by membrane deformation was stomatocytes. Briefly the membrane was folded on one side and normal central depression, characteristics of discocytes, appeared like a hole in the center of the cell with biconcave shape of the cell completely lost. This type transformed cell was designated stomatocyte, and they have been reported in various hemolytic disease.

About 2% to 3% of the RBC population of caffeine treated rat exhibit shapes and morphology similar to a ring. The characteristics feature of the cell was the presence of alternate bands of electron light and electron dense areas. The number of ring shaped cells in the erythrocyte from caffeine treated rat was very few, but a large
Plate 1. Scanning electron micrograph of some erythrocytes of rats
a. Normal discocytes in control group of rats x1500
b. Normal discocytes in control group of rats x3200
c. Abnormal erythrocytes in caffeine fed rats x1500
d. Tear drope shaped cell in caffeine fed rats x3200
e. Normal erythrocytes with some abnormal erythrocytes in caffeine and vitamin C fed rats x1500
f. Normal erythrocytes with some abnormal erythrocytes in caffeine and vitamin C fed rats x3200
number of erythrocytes showed abnormalities which were indicative of the early stages of ring shaped cell formation.

A few cells were similar in appearance to a tear drop, and these were regarded as abnormal cells. (Plate 1d)

The erythrocytes found in caffeine and simultaneously vitamin C treated rats were mainly normal discocytes although abnormal erythrocyte also appeared. The abnormalities such as echinocytogenesis, stomatocytogenesis, membrane internalization and ring shaped cells were reduced significantly. (Plate 1e, 1f)

**Scanning Electron Microscopic Examination of Organ**

Scanning electron microscopic examination of organ of kidney revealed external detail and also information regarding intercellular gap. Scanning electron microscopic investigation allows the surface structure of cell and other tissue component to be delineated. Excellent depth of focus of the SEM makes it possible to visualize surfaces of the intake cells in native configuration.
Plate 2. Scanning electron micrograph of kidney

a. Normal structure of kidney in control set of rats x3000
b. Abnormal structure of kidney in caffeine treated set of rats with loss of connective tissue x3000
c. Abnormal structure of kidney in caffeine and vitamin C supplemented set of rats with partial loss of connective tissue x3000
Scanning Electron Microscopic Examination of kidneys

Scanning Electron Microscopic Examination revealed glomerular capillary. The wall of the capillary showed horizontal ridge formed by the cytoplasm of the endothelial cells.

In caffeine treated rat, there was a decline in cytoplasmic ridges with loss of endothelial cell structure and tubular architecture. (Plate 2b)

In simultaneously caffeine and vitamin treated rat no remarkable structural change with caffeine treated rat was recorded except mild recovery of the caffeine induced structural damages. (Plate 2c)

Histology structure and pathological manifestation of organs

Liver

Control liver showed a normal architecture of liver histology. Hepatic cords were present and cells were intact with typical structure of hepatocytes. Sinusoidal space was within normal limits. (Plate 3a, 3b)

Caffeine treated rat liver showed marked changed. Loss of intercellular cementing material was also observed. Two or three cell thick trabeculi were formed. Most of the hepatocytes became larger than the normal size. Breakdown of cords were observed with separated cells. Swallowing of cells and karyolysis were observed. Liver
Plate 3. Photomicrograph of liver

a-b Control rat showing normal hepatic cord with hepatocytes
  a. Low magnification  x 200  b. High magnification  x 400

c-d Caffeine fed rat showing loss of cytoplasm in hepatocytes
  c. Low magnification  x 200  d. High magnification  x 400

e-f Caffeine and vitamin C fed rat showing loss of cytoplasmic material with some normal hepatocytes
  e. Low magnification  x 200  f. High magnification  x 400
sinusoids were wider than the normal and sinusoidal spaces were covered by cytoplasm of necrotic cells. Loss of cytoplasmic materials was commonly seen. Loss of nuclear materials were also seen in some areas. Multinucleated hepatocytes were also present but there was the evidence of regeneration of hepatocytes in the later part of the caffeine treatment, which indicated the condition of tolerance. Loss of nuclear material was also seen in some areas. Nuclear pleomorphism was comparatively slight. There was prominent central nuclei and most of them were larger than the normal nuclei. (Plate 3c, 3d)

In caffeine and ascorbic acid treated rat, hepatic cords were found without breakage while reappearing of intercellular cementing matrix was observed. Sinusoidal spaces were wider than normal and also occupied by the cytoplasm of some necrotic cells. No swallowing of cellular structure was found. The hepatocytes appeared to be a little normal structure after continuation of vitamin C supplementation while loss of some cytoplasmic materials was also commonly found. (Plate 3e, 3f)

**Thyroid gland**

Thyroid is an important endocrine gland that secretes hormone, which regulates the metabolic processes of the body. Histological examination revealed the structural detailed of the gland in control set of experimental rat and also histopathological changes in treated rat of both caffeine administered and also simultaneous caffeine and vitamin C supplemented group.
Plate 4 Photomicrograph of thyroid

**a-b** Control thyroid follicle showing colloidal material with active thyroid follicular cell, vaculation present

a. Low magnification  x 200  
b. High magnification  x 400

**c-d** Caffeine fed rat showing low amount of thyroglobulin without vaculation

c. Low magnification  x 200  
d. High magnification  x 400

**e-f** Caffeine and vitamin C fed rat showing active cells with vaculation in colloid

e. Low magnification  x 200  
f. High magnification  x 400
Control thyroid of normal rat contained large number of thyroid follicles that were separated by connective tissue sheath as seen. Each thyroid follicle is surrounded by very thin smooth basement lamina. Thyroid cell appeared to form a ring around the colloid. Another cell type, parafollicular cells were present either singly or in small groups. Vaculations in colloid with net like colloid material were also seen clearly. (Plate 4a, 4b)

In caffeine fed rat thyroid follicles were irregular, the follicular epithelial layer thin. The follicular colloids became less dense and more basophilic. Some follicles ruptured and coalesce forming colloid cyst. Vaculation in colloid was not seen which indicated loss of absorption of colloidal material or hormone by blood. Loss of eosinophilic material was commonly seen with eosinophilic stain. Most of the thyroid follicles were found to be separated from adjacent cells. Although solid follicles were present but most of thyroid follicles were empty and showed only the solid sheath of epithelial cells indicating the inhibition of thyroxin synthesis. (Plate 4c, 4d)
In case of Caffeine and vitamin C treated group of rat large number of thyroid follicles with irregular shape and size were observed. Most of the thyroid follicles were separated from adjacent parafollicular cells and connective tissue sheath. Vaculation was also present in follicular colloids, which indicated continued synthesis and absorption of colloidal material. The colloidal amount was high in vitamin C supplemented rat as compared to caffeine fed rat (Plate 4e, 4f).

Thyroid follicular cell size appeared to be more or less normal probably due to interference of vitamin C with toxic impact of caffeine in cellular level.

Kidney

In control rat kidney structure showed normal configuration with normal glomeruli and normal tubular pattern. Bowmen's capsule was also seen normal with visceral and parietal layer. Cellular necrosis was not observed (Plate 5a, 5b). Tubular structure also appeared as normal. (Plate 6a, 6b)

In caffeine fed rat, there was partial damaged glomeruli with infiltration of cells in Bowmens capsule. Glomerular atrophy was seen in some areas. Lesion's were confirmed to a part of each glomerulus while in some glomeruli had lesions. The foci of necrosis and disruption of capillary loops were present. Glomerular necrosis was also observed in some areas of the kidney. Cellular necrosis is prominent with loss of nucleus in medullary regions. The wall of the arteriole became thick. Dilatation of proximal convoluted tubule was also found in most of the nephrone. There was a diffuse
Plate 5 Photomicrography of kidney (cortex)

**a-b** Control rat showing normal glomerulus
a. Low magnification x 240  
  b. High magnification x 400

**c-d** Caffeine fed rat showing loss of cellular structure in glomerulus
  c. Low magnification x 240  
  d. High magnification x 400

**e-f** Caffeine and vitamin C fed rat showing some normal and some abnormal glomerulus
  e. Low magnification x 240  
  f. High magnification x 400
proliferative changes in the glomerulus, with increase in the number and size of the endothelial and mesangial cells. The mesangial cells were particularly marked and had produced diffused eosinophilic sclerosis of the glomerulus. The tubular structure of glomerulus was found to be obvious than normal while most of the cell were damaged.

(Plate 5c, 5d)

In caffeine and simultaneously vitamin C administered rat also showed pathological changes but a partial tendency of recovery was observed. The cellular structure of tubular regions also appeared normal (Plate 6e, 6f). Glomerular structure also indicated more or less normal configuration with necrotic changes and almost normal histopattern. Simultaneous administration of vitamin C appeared to interfere with the toxic effect of caffeine on renal tissue as indicted by the experiment.
Plate 6. Photomicrograph of kidney (Medulla)

a-b Control rat showing normal tubular structure with prominent nucleus
   a. Low magnification x 200     b. High magnification x 400

c-d Caffeine fed rat showing the loss of cellular structure with breakage of cell membrane and release of nucleus
   c. Low magnification x 200     d. High magnification x 400

e-f Caffeine and vitamin C treated rat showing partial recovery of cellular structure
   e. Low magnification x 200     f. High magnification x 400