CHAPTER - I

EFFECT OF LONG-TERM CONSUMPTION OF CAFFEINE ON THE DEVELOPMENT OF EHRlich ASCITES CARCINOMA (EAC) CELLS IN FEMALE MICE
INTRODUCTION

Caffeine has been considered as possible teratogen because of its similar structure as found in purine bases of deoxy ribonucleic acid (DNA) (80). Caffeine at doses of 0.05% in drinking water stimulates spontaneous mammary tumorigenesis in mice (221) and is able to accelerate pancreatic carcinogenesis in hamsters when administered during the post initiation phase of the tumor (222). Pituitary adenomas also observed in female rats after 12 months of caffeine administration at doses of 2g/l in drinking water (223). On the other hand, several authors have reported that caffeine antagonizes the carcinogenic effect of DNA damaging agents both under in vitro and in vivo conditions (266, 301). It has also been observed that caffeine significantly inhibits carcinogenesis in mouse skin induced by different chemical agents, including those related to cigarette smoke and tobacco (227). Though no definite or clear single molecular or cellular mechanism have been identified for the anticarcinogenic activity of caffeine, it plays its action either by direct binding to DNA or by alteration of DNA repair processes (206, 236, 302, 303). Inhibitory effect of caffeine on repair processes at the level of post replication repair synthesis of DNA (248) or poly (ADP-ribose) polymerase (252) have also been reported. An alternative mechanism of caffeine action in G2 phase (the interval between the end of DNA synthesis and the beginning of the mitosis) of cell cycle has been suggested. Caffeine in fact induces cells to undergo mitosis before the completion of DNA repair (252, 253). Further, it is known that caffeine enhances the tumorigenesis by altering (either positively or negatively) the neuroendocrine activity (218) depending on the time span of caffeine treatment, the dose of caffeine used etc. In fact, in rodents, caffeine affects (either increase or decrease) activities of brain catecholamines, serotonin, acetylcholine and γ-aminobutyric acid (GABA) as well as adenosine-mediated neurotransmission of the brain depending on dose and duration of caffeine treatment (304, 305). Altered activity of the central nervous system (CNS) by caffeine could in turn influence anterior pituitary hormone secretion (306). It has also been reported that in rodents, caffeine relatively at its high dose decreases serum levels of thyroid stimulating hormone, increases serum corticosterone levels and inhibits the pulsatile
secretion of growth hormone (306, 307). These altered secretory rates of these hormones, i.e. thyroid stimulating hormone, growth hormone and adrenal corticosterone have been reported to influence the development of neoplastic rodent mammary gland (308, 309). Thus, the role of caffeine in relation to carcinogenesis seems to be undefined.

The respiration of tumor cells has been considered as an index of viability (284). Tumor cells are known to exhibit an unusually high rate of aerobic glycolysis in comparison with normal cell (284). It has also been suggested that the energy necessary for survival and growth of tumor cells is derived from active glycolysis (310).

Further, the metabolic changes of the normal tissues of the tumor-bearing animal have been studied using the endocrine organs of the host in presence of a growing tumor. Corticosterone is known to be a major adrenal steroid in mice (267, 311, 312), the concentration of which in blood and adrenal is generally used to assess the adrenocortical function. It would also appear that a growing tumor induces an adrenal response like a nonspecific chronic stress (267). Therefore, corticosterone status is considered as one of the major index for measuring the stress-induced response. Begg and others (267, 268, 313) reported that during development of tumor growth, adrenal shows its hypofunctional state by decreasing adrenal osmophilia, reduction in ascorbic acid and cholesterol content to produce corticosterone. It is well known that rat adrenal is one of the richest sources of ascorbic acid (314). Administration of ascorbic acid has been found to reduce the production of corticosterone in response to adrenocorticotropic hormone (ACTH) administration indicating that ACTH stimulates corticosterone biosynthesis by decreasing adrenal ascorbate levels (315). Recent past epidemiological studies indicates that ascorbic acid is protective against cancer (316, 317).

Considering all those informations, we are interested in the present investigation to study the effect of caffeine in the development of Ehrlich ascites carcinoma (EAC) cells at the levels of corticosterone status, adrenal ascorbic acid, the respiration and viability of the EAC cells and $[^{3}H]$- thymidine incorporation in the EAC cells.
MATERIALS AND METHODS

Chemicals
Caffeine was purchased from Fluka - Chemica - Biochemica (Switzerland). Corticosterone and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [3H]-thymidine (Sp. activity 6.5 Ci / mmole) was purchased from Board of Radiation and Isotope Technology, Bombay, India. All other reagents used in the present study were of analytical grade and they are described in detail in 'General Materials and Methods'.

Experimental Animals
Adult female swiss mice (20-25 gm body weight), kept in a 12 h dark-12 h light cycle at room temperature (28° ± 0.5°C) with constant relative humidity (80 ± 5%) were maintained with standard laboratory diet and water ad libitum.

Transplantation of EAC cells
The EAC cells were transplanted into recipient mice by intraperitoneal inoculation with 0.2 ml of ascites fluid containing 10^7 cells (approx.) obtained from the tumor bearing mice. This cell concentration of ascites fluid was made up with sterile normal saline (284).

Treatment of caffeine in mice with or without EAC cells
Mice were divided into 7 groups. Each group was divided into three subgroups a, b, c. Each subgroup contains 4-5 animals. Animals of groups 2a, 2b and 2c were treated with caffeine (20 mg / kg / day p.o) in a volume of 0.2 ml for 24, 27 and 30 consecutive days respectively. Animals of subgroups 1a, 1b, and 1c were treated with an equal volume of vehicle (water) of caffeine through the same route for the same period under similar conditions. The subgroups 1a, 1b and 1c were considered as control of subgroups of experimental animals 2a, 2b and 2c respectively. Animals of subgroups 4a, 4b and 4c were the recipients of EAC cells. The EAC cells (0.2 ml) were transplanted intraperitoneally (i.p) to these three subgroups (4a, 4b and 4c) of
mice and they were allowed to develop EAC cells for 12, 15 and 18 days respectively. Animals of subgroups 3a, 3b and 3c were treated with 0.2 ml of saline through the same route (i.p) under similar conditions and they were considered as control of subgroups 4a, 4b and 4c respectively. The animals of subgroups 6a, 6b and 6c were inoculated intraperitoneally with EAC cells after 12 consecutive days of treatment with vehicle (water) of caffeine and the treatment was continued for another 12, 15 and 18 consecutive days respectively. Animals of subgroups 7a, 7b and 7c were inoculated intraperitoneally with EAC cells after 12 consecutive days of caffeine treatment and the caffeine treatment of the subgroups 7a, 7b and 7c were continued for another 12, 15 and 18 consecutive days respectively. Animals of subgroups 5a, 5b and 5c were treated (i.p) with 0.2 ml saline (as vehicle of EAC cells) after treatment with an equal volume of vehicle (water) of caffeine through the same route for 12 consecutive days which was continued for another 12, 15 and 18 consecutive days as did in experimental groups and were considered as control of corresponding experimental subgroups 6a, 6b and 6c respectively as well as subgroups 7a, 7b and 7c respectively. Conditions of these treatment are also represented in a flow sheet (Fig 5) in 'General Materials and Methods'.

**Collection of tissues and cells**

Mice of both control and experimental groups were killed by cervical dislocation. Caffeine treated animals were killed 30 min after the last caffeine administration. Adrenals were immediately taken out and collected in 0.1 (N) HCl and 5% meta-phosphoric acid for the estimation of corticosterone and ascorbic acid respectively. Blood was collected with heparin as an anticoagulant and plasma was prepared according to the method as described by Talwar (279). EAC cells were collected from peritoneum cavity. The details of the methods are described in 'General Materials and Methods'.

**Biochemical assay**

EAC cells were collected from the peritoneum cavity of the cancerous mice. The cells were initially diluted and washed with normal saline and the pellets were
finally washed with and resuspended in PBS and were used for the estimation of cell viability, oxygen consumption and $[^{3}H]$-thymidine incorporation. The EAC cell viability was estimated according to the method of Bekesi et al. (283). Oxygen consumption of EAC cells was measured following the method of Ray et al. (284). $[^{3}H]$-thymidine incorporation to the EAC cells was measured following the method of Kupka et al. (285). Corticosterone level in plasma and adrenal gland was estimated spectrofluorometrically following the method of Purves and Sierett (286) and Vernikoss-Daniellis et al. (287) respectively. Level of total ascorbic acid in adrenal gland was estimated spectrophotometrically following the method of Roe and Kuether (288). Reduced vitamin C in adrenal gland was estimated according to the method of Glick (289). Protein was estimated by following the method of Lowry et al. (299) using BSA as standard. The details of all the methods are described in 'General Materials and Methods'.

**Statistical Analysis**

The statistical significance between the control and experimental values were assessed by analysis of variance (ANOVA) using Tukey test (300).

**RESULTS**

Table 6 appears to show that during the development of EAC cells, the cell viability (49.28%; $F = 1181.00$, df = 1.6; $P < 0.025$), oxygen consumption (53.47%; $F = 288.39$, df = 1.6; $P < 0.025$) and $[^{3}H]$-thymidine incorporation (57.13%; $F = 10.59$, df = 1.6; $P < 0.025$) were significantly increased on 18th days of development with respect to that observed on 12th days of development of EAC cells.

It is evident from Table 7 that the cell viability of EAC cells was significantly reduced when mice were pretreated with caffeine for 12 consecutive days and continued for another 15 (46.1%; $F = 32.67$, df = 1.6; $P < 0.05$) and 18 (81.44%; $F = 282.31$, df = 1.6; $P < 0.05$) consecutive days after inoculation of EAC cells with respect to the development of EAC cells in presence of vehicle of caffeine. Oxygen
Table 6: Estimation of cell viability, oxygen consumption and $[^3H]$-thymidine incorporation in EAC cells.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Duration of development of EAC cells (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Cell viability ($\times 10^6$ cell / ml)</td>
<td>6.94 ± 0.13</td>
</tr>
<tr>
<td>Oxygen consumption (natom oxygen consumed / sec / ml)</td>
<td>17.00 ± 0.50</td>
</tr>
<tr>
<td>$[^3H]$-thymidine incorporation (pmole / 10^7 cells / ml)</td>
<td>47.56 ± 3.75</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM of 4-5 separate observations.

EAC cells were transplanted intraperitoneally from Ehrlich ascites tumor bearing mice to recipient mice.

Significantly different *P < 0.025 using Tukey test for ANOVA.
Table 7: Estimation of cell viability, oxygen consumption and $[^3]$H-thymidine incorporation following long-term caffeine consumption during the development of EAC cells.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Duration of development of EAC cells / period of vehicle of caffeine treatment (days)</th>
<th>Duration of development of EAC cells / period of caffeine treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 (24)*</td>
<td>15 (27)*</td>
</tr>
<tr>
<td>Cell viability $(\times 10^6 \text{cell/ml})$</td>
<td>7.06 ± 0.56</td>
<td>8.07 ± 0.50</td>
</tr>
<tr>
<td>Oxygen consumption $(\text{natom of oxygen consumed}/\text{sec/ml})$</td>
<td>17.38 ± 0.81</td>
<td>20.07 ± 1.21</td>
</tr>
<tr>
<td>$[^3]$H-thymidine incorporation $(\text{pmole/}10^7\text{cells/ml})$</td>
<td>47.94 ± 2.69</td>
<td>55.50 ± 2.07</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 4-5 separate observations.

EAC cells were transplanted intraperitoneally from EAC cell-bearing mice to recipient mice which were pretreated with caffeine for 12 consecutive days and continued for another 12, 15 or 18 consecutive days after the inoculation of EAC cells.

$^a$Number in the parenthesis indicates the duration of caffeine or its vehicle treatment.

Significantly different *$P < 0.05$ using Tukey test for ANOVA.
consumption as well as incorporation of $[^3]$H-thymidine in EAC cells were also significantly reduced when mice were pretreated with caffeine for 12 consecutive days and continued for another 15 (49.45%; $F = 47.60$, df = 1,6; $P < 0.05$ and 46.68%; $F = 16.31$, df = 1,6; $P < 0.05$ respectively) and 18 (79.63%; $F = 8073.39$, df = 1,6; $P < 0.05$ and 79.25%; $F = 46.67$, df = 1,6; $P < 0.05$ respectively) consecutive days following inoculation of EAC cells with respect to the development of EAC cells in presence of vehicle of caffeine under similar conditions. Whereas, pretreatment of caffeine for 12 consecutive days followed by another 12 consecutive days of caffeine treatment along with the inoculation of EAC cells showed an apparent reduction in those parameters of EAC cells with respect to the development of EAC cells in presence of vehicle of caffeine under similar conditions. Further, it is noted that the administration of caffeine along with the inoculation of EAC cells significantly reduced the cell viability, oxygen consumption and $[^3]$H-thymidine incorporation following 15 (29.04%; $F = 7.20$, df = 1,6; $P < 0.05$; 32.13%; $F = 13.99$, df = 1,6; $P < 0.05$; 25.65%; $F = 7.71$, df = 1,6; $P < 0.05$ respectively) and 18 (68.35%; $F = 110.81$, df = 1,6; $P < 0.05$; 64.33%; $F = 264.54$, df = 1,6; $P < 0.05$; 61.51%; $F = 36.34$, df = 1,6; $P < 0.05$ respectively) consecutive days of caffeine treatment in comparison to that observed with 24 consecutive days of caffeine treated condition.

Figs. 6 and 7 depicts that corticosterone level in plasma and in adrenal gland remain unchanged when mice were treated with caffeine alone for 24, 27 or 30 consecutive days. Fig. 6 also demonstrates that plasma corticosterone level was increased during the development of EAC cells for 12 (88.97%; $F = 48.90$, df = 1,8; $P < 0.025$), 15 (91.89%; $F = 322.57$, df = 1,8, $P < 0.025$) and 18 (98.58%; $F = 1681.13$, df = 1,8; $P < 0.025$) days with respect to their corresponding control. But the corticosterone level in adrenal gland was significantly decreased during the development of EAC cells for 12 (82.14%; $F = 94.43$, df = 1,8; $P < 0.025$), 15 (62.07%; $F = 369.50$, df = 1,8; $P < 0.025$) and 18 (44.44%; $F = 276.92$, df = 1,8; $P < 0.025$) days with respect to their corresponding control (Fig. 7). Further, continuation of development of EAC cells for 18 days significantly increased adrenal corticosterone level (200%; $F = 270.00$, df = 1,8; $P < 0.025$) with respect to 12 days development of EAC cells (Fig. 7). The corticosterone level in plasma and in adrenal
Fig. 6: Effect of long-term consumption of caffeine on EAC cell-induced changes in corticosterone level of plasma.

Results are expressed as mean ± SEM of 4-5 separate observations.

Vertical line on top of each bar indicates ± SEM.

No significant difference between the controls of the corresponding experimental conditions.

Control value of corticosterone level in plasma (µg / ml) is 0.21 ± 0.04

Caffeine (20mg / kg / day) was administered (p.o) to mice for 24 (1), 27 (2) and 30 (3) consecutive days respectively and corresponding control group was treated with vehicle (water) of caffeine under similar conditions.

EAC cell after transplantation (i.p) were allowed to develop for 12 (4), 15 (5) and 18 (6) days. The corresponding control group was injected with 0.2 ml of saline under similar conditions.

EAC cells were transplanted (i.p) to mice which were pretreated with vehicle (water) of caffeine for 12 consecutive days and continued for another 12 (7), 15 (8) and 18 (9) consecutive days. The corresponding control group was injected with 0.2 ml saline as vehicle of EAC cells under similar conditions.

EAC cells were transplanted (i.p) to mice which were pretreated with caffeine for 12 consecutive days and continued for another 12 (10), 15 (11) and 18 (12) consecutive days. Control groups corresponding to experimental groups was treated with vehicle (water) of caffeine along with the injection of 0.2 ml saline under similar conditions.

Significantly different mP < 0.025 using Tukey test for ANOVA.
Fig. 7: Effect of long-term consumption of caffeine on EAC cell-induced changes in corticosterone level of adrenal gland. 

Results are expressed as mean ± SEM of 4-5 separate observations. Vertical line on top of each bar indicates ± SEM. No significant difference between the controls of the corresponding experimental conditions. Control value of corticosterone level in adrenal (µg / mg protein) is 0.28 ± 0.003. Other details are same as described in Fig. 6. Significantly different *P < 0.025 using Tukey test for ANOVA.
gland remain unchanged, when mice were pretreated with caffeine for 12 consecutive
days and continued for another 12, 15 or 18 consecutive days after inoculation of the
mice with EAC cells with respect to their corresponding control mice. It is also
observed from this figure (Fig. 6) that the EAC cell-induced increase in plasma
corticosterone level was significantly reduced when caffeine was administered for 24
(55.75%; $F = 53.89, df = 1,8; P < 0.025$), 27 (48.8%; $F = 43.22, df = 1,8; P < 0.025$)
and 30 (40.86%; $F = 587.34, df = 1,8; P < 0.025$) consecutive days, including 12
consecutive days of caffeine treatment prior to EAC cell inoculation with respect to
the EAC cell developing conditions alone. Whereas, the corticosterone level in
adrenal gland was increased when caffeine was treated for 12 consecutive days prior
to EAC cell inoculation and its treatment was continued for 12 (250%; $F = 53.33,
df = 1,8; P < 0.025$), 15 (100%; $F = 9.73, df = 1,8; P < 0.025$) and 18 (60%;
$F = 252.50, df = 1,8; P < 0.025$) consecutive days with respect to corresponding EAC
cell developing conditions alone (Fig. 7). During the development of EAC cells for 15
days, corticosterone level in adrenal gland was significantly enhanced (120%;
$F = 90.00, df = 1,8; P < 0.025$) with respect to the development of EAC cells for 12
days following its inoculation.

It is evident from Table 8 that treatment with caffeine for 24, 27 and 30
consecutive days did not significantly changed the total as well as reduced adrenal
ascorbic acid levels with respect to their corresponding control.

Table 9 appears to show that the total adrenal ascorbic acid level was
significantly decreased when EAC cells were developed for 12 (28.44%; $F = 474.93,
df = 1,8; P < 0.001$), 15 (31.56%; $F = 155.02, df = 1,8; P < 0.001$) and 18 (44.68%;
$F = 794.88, df = 1,8; P < 0.001$) days with respect to their corresponding control. The
development of EAC cells for 12 (39.01%; $F = 77.32, df = 1,8; P < 0.001$), 15
(43.12%; $F = 1148.37, df = 1,8; P < 0.001$) and 18 (45.09%; $F = 516.92, df = 1,8;
P < 0.001$) days also decreased the reduced ascorbic acid level with respect to their
 corresponding control.

It appears from Table 10 that no significant change was observed in adrenal
ascorbic acid (both total and reduced) levels when caffeine was administered for 12
consecutive days prior to the inoculation of EAC cells and continued during the
Table 8: Effect of long-term consumption of caffeine on total and reduced ascorbate levels in adrenal gland of mice

<table>
<thead>
<tr>
<th>Period of caffeine treatment (days)</th>
<th>Adrenal ascorbic acid level</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (µg / mg protein)</td>
<td>Reduced (µg / mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>20.96 ± 0.98</td>
<td>23.36 ± 1.71</td>
<td>10.21 ± 0.06</td>
<td>10.74 ± 0.03</td>
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</tr>
<tr>
<td>27</td>
<td>22.10 ± 1.11</td>
<td>26.48 ± 2.07</td>
<td>11.32 ± 0.12</td>
<td>11.30 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>21.21 ± 2.21</td>
<td>23.39 ± 2.01</td>
<td>11.33 ± 0.24</td>
<td>12.19 ± 0.50</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 4-5 separate observations.
Caffeine was administered (p.o) to mice at a dose of 20 mg / kg / day and corresponding control groups were treated with vehicle (water) of caffeine under similar conditions.
Table 9: Effect of the development of EAC cells on the level of total and reduced ascorbic acid in adrenal gland of mice.

<table>
<thead>
<tr>
<th>Duration of EAC cell development (days)</th>
<th>Adrenal ascorbic acid level</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (μg / mg protein)</td>
<td>Reduced (μg / mg protein)</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>21.45 ± 0.63</td>
<td>15.35 ± 0.48*</td>
<td>12.92 ± 0.04</td>
<td>7.88 ± 0.05*</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>20.66 ± 1.71</td>
<td>14.14 ± 0.55*</td>
<td>13.01 ± 0.03</td>
<td>7.40 ± 0.18*</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>22.27 ± 0.91</td>
<td>12.32 ± 0.77*</td>
<td>13.13 ± 0.05</td>
<td>7.21 ± 0.19*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 4-5 separate observations.
EAC cells (≈10^7 cell) in 0.2 ml were transplanted intraperitoneally from EAC cell bearing mice to recipient mice. The corresponding control group was injected with 0.2 ml saline under similar conditions.
Significantly different *P < 0.001 using Tukey test for ANOVA.
Table 10: Effect of long-term consumption of caffeine on EAC cell-induced changes in total and reduced ascorbic acid level of mice adrenal gland.

<table>
<thead>
<tr>
<th>Experimental condition(s)</th>
<th>Duration of development of EAC cells / period of caffeine or its vehicle treatment (days)</th>
<th>Ascorbic acid level</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (µg / mg protein)</td>
<td>Reduced (µg /mg protein)</td>
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<td></td>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>Development of EAC cells in presence of caffeine</td>
<td>12 (24)§</td>
<td>21.45 ± 0.63</td>
<td>15.95 ± 0.75*</td>
<td>12.92 ± 0.04</td>
<td>7.95 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>15 (27)§</td>
<td>20.66 ± 1.71</td>
<td>15.11 ± 0.89*</td>
<td>13.01 ± 0.03</td>
<td>7.77 ± 0.27*</td>
</tr>
<tr>
<td></td>
<td>18 (30)§</td>
<td>22.27 ± 0.91</td>
<td>12.83 ± 0.21*</td>
<td>13.13 ± 0.05</td>
<td>7.38 ± 0.13*</td>
</tr>
<tr>
<td>Development of EAC cells in presence of caffeine</td>
<td>12 (24)§</td>
<td>21.79 ± 2.10</td>
<td>23.25 ± 2.35*</td>
<td>10.92 ± 0.07</td>
<td>9.76 ± 0.08*</td>
</tr>
<tr>
<td></td>
<td>15 (27)§</td>
<td>22.98 ± 1.69</td>
<td>23.52 ± 1.55*</td>
<td>10.12 ± 0.10</td>
<td>10.26 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>18 (30)§</td>
<td>20.43 ± 1.22</td>
<td>20.22 ± 0.35*</td>
<td>10.76 ± 0.05</td>
<td>10.71 ± 0.21*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 4-5 separate observations.

*EAC cells were transplanted intraperitoneally from EAC cell-bearing mice to recipient mice which were pretreated with vehicle (water) of caffeine for 12 consecutive days and continued for another 12, 15 and 18 consecutive days. The corresponding control group was injected with 0.2 ml saline under similar conditions.

The mice were pretreated with caffeine for 12 consecutive days and continued for another 12, 15 and 18 consecutive days after the inoculation of EAC cells. The corresponding control group was treated with vehicle (water) of caffeine along with the injection of 0.2 ml saline under similar conditions.

*Number in the parenthesis indicates the total duration of caffeine or its vehicle treatment.

Significantly different *P < 0.005 using Tukey test for ANOVA.
development of EAC cells for 12, 15 and 18 consecutive days with respect to their corresponding control as well as with respect to the corresponding only caffeine treated conditions (Table 8). Table 10 also demonstrates that the EAC cell-induced decrease in total adrenal ascorbic acid level was restored to the control level when caffeine was administered for 24 (51.47%; \( F = 18.74, \) df = 1,8; \( P < 0.005 \)), 27 (66.348%; \( F = 54.05, \) df = 1,8; \( P < 0.005 \)) and 30 (57.6%; \( F = 350.62, \) df = 1,8; \( P < 0.005 \)) consecutive days, including 12 consecutive days of caffeine treatment prior to EAC cell inoculation with respect to the corresponding EAC cell developing conditions alone (Table 9). Similarly, the reduced ascorbic acid level in adrenal gland was significantly increased when caffeine was treated for 12 consecutive days prior to EAC cell inoculation and its treatment was continued for 12 (23.86%; \( F = 378.42, \) df = 1,8; \( P < 0.005 \)), 15 (38.65%; \( F = 200.31, \) df = 1,8; \( P < 0.005 \)) and 18 (41.11%; \( F = 156.10, \) df = 1,8; \( P < 0.005 \)) consecutive days with respect to the corresponding EAC cell developing conditions alone (Table 9).

DISCUSSION

Caffeine, one of the methyl xanthine (MXs) compounds, is known to be an alkaloid in nature (3). Because of the wide spread and time immemorial use of caffeine, a number of epidemiological studies have been carried out at the different levels with respect to different diseases including neoplastic disease for the last several years to determine the action of caffeine in relation to its doses use and duration of treatment. Caffeine has been reported to inhibit phosphodiesterase activity (158), affect anterior pituitary gland secretion (306), the activation of CNS neurotransmitter (304), serum fatty acid levels (318), intracellular calcium transport (319) and also known to be an adenosine receptors antagonist (159, 175, 177). Any number of these physiological / biochemical events, singly or in combination, could influence tumor development and / or its growth processes. But no consistent observations and its defined mechanism of actions have been underlined. In view of these informations, a systemic attempt has been made in the present investigation to
study the effect of caffeine in relation to the development of EAC cells with respect to (a) the viability of EAC cells in terms of respiration and $[^{3}H]$-thymidine incorporation in tumor cells, (b) the status of corticosterone, a major adrenal steroid and the changes in adrenal ascorbic acid, if any.

The results of the present study demonstrates that long-term caffeine treatment suppresses the EAC cells growth, which has also been found to be dependent on the time span of caffeine treatment (Table 7). Caffeine-induced suppression of EAC cell growth may be supported by the works of Theiss et al. (301) and others (227, 235, 236, 320) who in fact have shown that caffeine suppresses both spontaneous and chemically-induced pulmonary adenoma development, apoptosis and cell cycle.

Plasma corticosterone level in the present study has been found to be elevated with a decrease of its adrenal level during the development of EAC cells (Figs. 6 and 7). This may be explained by the fact that the secretion of corticosterone in plasma during tumor growth may be much greater than its synthesis in adrenal (Figs. 6 and 7) possibly due to the hyperactivity of the adrenal gland (267). The growth of EAC cells during long-term caffeine treatment on the other hand, shows no appreciable change in the level of both plasma and adrenal corticosterone with respect to their corresponding control (Figs. 6 and 7) suggesting that long-term caffeine treatment may have a negative role over EAC cell growth. Long-term caffeine consumption, which has been found to develop tolerance to caffeine (123, 126) does not appreciably change the corticosterone status (Figs. 6 and 7).

The hypersecretion of ACTH causes an increase of plasma corticosterone level as well as tumor growth (321). Further, it is known that increased secretion of ACTH results in the depletion of adrenal ascorbic acid by hydrolytic decomposition of dehydroascorbic acid to 2, 3 - diketogulonate (322). Thus, the reduced level of adrenal ascorbic acid (both total and dehydro) observed in the present study (Table 9) during the development of EAC cells suggests that EAC cell development enhances ACTH secretion followed by elevation of plasma corticosterone level and reduction of adrenal ascorbic acid level (Fig. 6 and Table 9). Though long-term caffeine treatment (which generally produces tolerance to caffeine) did not show any significant change in the level of corticosterone as well as reduced and total ascorbic acid in adrenal
(Figs. 6 and 7 and Table 8), the continuation of caffeine treatment during EAC cell development suppresses the EAC cell-induced induction of plasma corticosterone level (Fig. 6) and enhances the EAC cell-induced reduction of adrenal ascorbic acid level (Table 10) suggesting that long-term caffeine treatment may resist the EAC cell growth-induced changes at the level of both adrenal ascorbate and corticosterone status. In other words, long-term caffeine treatment may suppress the growth of EAC cells. Further, it may be assumed that development of EAC cell resist the long-term caffeine-induced development of tolerance to locomotor activity (LA) in mice (323).

Further, it may be mentioned that ACTH is a well known cyclic adenosine 3', 5'-monophosphate (cAMP) inducer through the stimulation of adenylate cyclase activity (324). But it is unlikely that induction of cAMP helps to produce the tumor growth, rather it is known that lowering of cAMP enhances the tumor growth (325). This phenomenon may be explained by the fact that ACTH-induced induction of intracellular cAMP induces the phosphodiesterase activity (326), cAMP degrading enzyme (327), as a result the cAMP level in the system may be down regulated and hence may stimulate the EAC cells growth in the present experimental condition. Now the question is, how does caffeine inhibit the EAC cell growth? This may be explained by the fact that caffeine is a well known phosphodiesterase inhibitor (156, 158). Thus, inhibition of this enzyme may stimulate/upregulate the cAMP level in the cellular environment and may reduce the EAC cell growth. Therefore, caffeine may inhibit or delay mitosis in many kind of cells, probably due to variations in cAMP concentration that influence DNA synthesis and mitosis (263). Again, it may also be explained by the fact that caffeine is a potent antioxidant (278), because it is an effective inhibitor of lipid peroxidation (LP) (277). This antioxidant property of caffeine may reduce the EAC cell growth.

Finally, it may be concluded from the present study that long-term pretreatment of caffeine and continuation of its treatment during EAC cell growth may (a) inhibit the growth of EAC cells (b) restore and modulate the EAC cell growth-induced changes in adrenal ascorbate and corticosterone status.