Caffeine inhibits the development of Ehrlich ascites carcinoma cells in female mice

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Long-term administration of caffeine at a dose of 20 mg/kg/day p.o. suppressed the viability, oxygen consumption and [3H]-thymidine incorporation of Ehrlich ascites carcinoma (EAC) cells. Though no significant change in the levels of plasma and adrenal corticosterone as well as both total and reduced adrenal ascorbic acid were observed following long-term caffeine consumption, pretreatment of caffeine and continuation of its treatment in the course of development of EAC cells restored the EAC Cell-induced changes in both corticosterone and ascorbic acid levels to control values. These results, thus, suggest that caffeine may suppress the growth of EAC cells by modulating the adrenal ascorbate level as well as corticosterone status.

Caffeine (1,3,7-trimethyl xanthine) due to its structural similarity with purine bases of DNA, has been considered as a possible teratogen. Chronic caffeine consumption significantly modulates by either potentiating or inhibiting the development of chemically-induced mammary carcinomas in female rats, lung tumorigenesis, skin carcinoma and uv-induced carcinoma. Caffeine shows its anticarcinogenic action either by direct binding to DNA or by inhibition of DNA repair processes. Anticarcinogenic effect of caffeine has been suggested by potentiation of the lethal effects of chemical carcinogens, leading to the death of cells that would have produced a tumor as a result of caffeine-sensitive post replication repair processes in cells. An alternative mechanism of caffeine action in G2 phase of cell cycle has also been proposed. Earlier observations of Kesavan et al., Raghu and Kesavan and others have shown the radioprotective and antioxidant effect of caffeine. There are some references where caffeine has been identified as a nonpotential genotoxic, mutagenic or carcinogenic agent. Further, it is reported that caffeine has no mutagenic risk in mammals including humans because of its short half life and rapid clearance in mammalian system. Thus, the role of caffeine in relation to carcinogenesis still remains to be identified. A systemic study is necessary to draw a clear understanding about the effect of long-term exposures to caffeine and coffee in mammals, including humans.

Corticosterone a major adrenal steroid is known to be an index of stress-induced response. Hilf et al., have also found that a growing tumor induces an adrenal response like a nonspecific chronic stress. The concentration of corticosterone in the blood and adrenal is generally considered a marker of adrenocortical function. Begg and others, way back in 1951 reported that during tumor growth in mice and rats a hypofunctional state is developed in the adrenal (known to be a rich source of ascorbic acid in mice) by decreasing adrenal osmophilia, reduction in ascorbic acid and cholesterol content for the formation of corticosterone. It is well known that corticosterone biosynthesis is stimulated by adrenocorticotropic hormone (ACTH) via reduction of adrenal ascorbate levels. Henson et al. and Padi in their epidemiological studies further, indicate that ascorbic acid is protective against cancer.

These informations led the authors to study the effect of long-term consumption of caffeine in the development of Ehrlich ascites carcinoma (EAC) cells in mice at the levels of the changes of its respiration, viability, [3H]-thymidine incorporation and corticosterone status as well as adrenal ascorbic acid.
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 (specific activity 6.5Ci/mmol) was purchased from Board of Radiation and Isotope Technology, Bombay, India. All other reagents used in the present study were of analytical grade.

Experimental Animals — Adult female Swiss mice (20-25g body weight), kept in a 12hr dark-12hr light cycle at room temperature (28°C ± 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 from donor mice into recipient mice by intraperitoneal inoculation with 0.2 ml of ascites fluid containing 10^7 cells (approx). This cell concentration of ascites fluid from donor mice was made up with sterile normal saline.

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 contained 4-5 animals. Animals of group 2a, 2b and 2c were treated with caffeine (20mg/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 as described in animals of groups 2a-2c. The subgroups 1a, 1b, 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 as described in animals of groups 4a-4c and they were considered as control of subgroups 4a, 4b and 4c respectively. Animals of subgroups 6a, 6b and 6c were inoculated intraperitoneally with EAC cells after 12 consecutive days of caffeine treatment and the caffeine treatment of the subgroups 6a, 6b and 6c were continued for another 12, 15 and 18 consecutive days respectively. The animals of subgroups 7a, 7b and 7c 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 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. These were considered as control for experimental subgroups 6a, 6b and 6c as well as subgroups 7a, 7b and 7c respectively.

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% m-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 described by Talwar. EAC cells were collected from peritoneal cavity.

Biochemical assay — EAC cells were collected from the peritoneal cavity of the experimental mice. The cells were initially diluted and washed with normal saline and the pellets were finally washed with and resuspended in PBS (phosphate buffered saline containing 40 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH was finally adjusted to 7.3 with 0.01 (N) NaOH solution) buffer and were used for the estimation of cell viability, oxygen consumption and [3H]-thymidine incorporation. The EAC cell viability was estimated according to the Trypan blue exclusion method of Bekesi et al. Oxygen consumption of EAC cells present in a reaction mixture containing PBS and approximately 10^7 cells was measured over a period of 10 min in an oxygraph (Gilson, villers le bel, France) equipped with a Clark electrode according to the method of Ray et al. [3H]-thymidine incorporation to the EAC cells was measured following the method of Kupka et al. In this method cell suspension was incubated in a total volume of 1 ml containing PBS, 0.1 mg (8.5 units) heparin and 1 mg glucose with 0.1μCi [3H]-Thymidine (specific activity 6.5 Ci/mmol) for 30 min at 37°C. The cells were immediately centrifuged at 12,000 g under 0-4°C and the pellet were suspended in 1 ml of 5% TCA and collected on
cellulose nitrate filter. Then counting was taken in a liquid scintillation counter (model no. 1209 Rackbeta). Corticosterone level in plasma and adrenal gland was estimated spectrophotometrically following the method of Vernikoss-Daniellis et al. Level of total ascorbic acid in adrenal gland was estimated spectrophotometrically following the method of Glick and Biskind. Protein was estimated by following the method of Lowry et al. Using bovine serum albumin as standard.

Statistical Analysis—The statistical significance between the control and experimental values were assessed by analysis of variance (ANOVA).

Results

Table 1 appears to show that during the development of EAC cells, the cell viability (49.28%), oxygen consumption (53.47%) and [H]-thymidine incorporation (57.13%) were significantly increased on the 18th day of development with respect to that observed on the 12th day of development of EAC cells. 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%) and 18 (81.44%) consecutive days after inoculation of EAC cells with respect to the development of EAC cells in presence of vehicle of caffeine. Oxygen consumption as well as incorporation of [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% and 46.68%) and 18 (79.63% and 79.25%) days following inoculation of EAC cells. Further, values obtained in the parameters studied after 27/30 days of treatment were found to be significantly lower than that observed at the end of 24 days consecutive treatment with caffeine.

Fig. 1(a) demonstrates that plasma corticosterone level was increased during the development of EAC cells for 12 (88.97%), 15 (91.89%) and 18 (98.58%) days with respect to their corresponding control. But in case of adrenal gland this corticosterone level was

<table>
<thead>
<tr>
<th>Experimental Condition (s)</th>
<th>Duration of Development (days)</th>
<th>Parameters</th>
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<tr>
<td></td>
<td>Cell viability (×10⁶ cell/ml)</td>
<td>Oxygen consumption (natom oxygen consumed/sec/ml)</td>
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<tr>
<td>Development of EAC Cells⁵</td>
<td>12</td>
<td>6.94 ± 0.13</td>
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<tr>
<td></td>
<td>15</td>
<td>8.14 ± 0.36</td>
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<td></td>
<td>18</td>
<td>10.36 ± 0.18*</td>
</tr>
<tr>
<td>Development of EAC Cells in presence of Vehicle of caffeine⁶</td>
<td>12 (24)⁷</td>
<td>7.06 ± 0.56</td>
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<tr>
<td></td>
<td>15 (27)⁷</td>
<td>8.07 ± 0.50</td>
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<td></td>
<td>18 (30)⁷</td>
<td>10.45 ± 0.36*</td>
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<tr>
<td>Development of EAC Cells in presence of Caffeine⁷</td>
<td>12 (24)⁷</td>
<td>6.13 ± 0.40</td>
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<td></td>
<td>15 (27)⁷</td>
<td>4.35 ± 0.53*</td>
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<tr>
<td></td>
<td>18 (30)⁷</td>
<td>1.94 ± 0.10*</td>
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Note: Values are expressed as mean ± SE of 4 separate observations.

⁵EAC cells were transplanted intraperitoneally from donors to recipients mice and the cells were allowed to develop for 12, 15 and 18 days.

⁶EAC cells were transplanted intraperitoneally from donors to recipients mice which were pretreated with vehicle (water) of caffeine for 12 consecutive days and continued for 12, 15 and 18 consecutive days.

⁷The 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.

²Number in the parentheses indicates the duration of caffeine or its vehicle treatment.

Significantly different from EAC cell developing conditions *P<0.05 using Tukey test for ANOVA.
Fig. 1 — Effect of long-term consumption of caffeine on EAC cell-induced changes in (a) corticosterone level of plasma and adrenal gland as well as (b) total and reduced ascorbic acid level in adrenal.

Results are expressed as mean ± SE of 5 separate observations. Vertical line on top of each bar indicates ± SE. No significant difference between the controls of the corresponding experimental conditions. Control value of corticosterone level in adren al (μg/mg protein) and plasma (μg/dl) are 0.28 ± 0.003 and 21.17 ± 0.35 respectively. Control value of total adrenal ascorbic acid level (μg/mg protein) and reduced ascorbic acid level (μg/mg protein) are 21.52 ± 0.23 and 11.90 ± 0.35 respectively. Caffeine (20 mg/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 cells 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 of 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 group corresponding to experimental group was treated with vehicle (water) of caffeine along with the injection of 0.2 ml saline under similar conditions. Significantly different *p<0.025, **p<0.005 using Tukey test for ANOVA.

significantly decreased by 82.14%, 62.07% and 44.44% respectively with respect to their control. The corticosterone level in plasma and in adrenal 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. It is also observed from Fig. 1(a) that the EAC cell-induced increase in plasma corticosterone level was significantly reduced when caffeine was administered
for 24 (55.75%), 27 (48.8%) and 30 (40.86%) consecutive days, including 12 consecutive days of caffeine treatment prior to EAC cell inoculation. Whereas, the corticosterone level in adrenal gland was increased under similar condition by 250%, 100% and 60% respectively with respect to corresponding EAC cell developing conditions alone. During the development of EAC cells for 15 days, corticosterone level in adrenal gland was significantly enhanced (120%) with respect to the development of EAC cells for 12 days following its inoculation. Further, continuation of development of EAC cells for 18 days significantly increased adrenal corticosterone level (200%) with respect to 12 days development of EAC cells.

It is evident from Fig. 1(b) that the treatment with caffeine did not significantly change the total as well as reduced ascorbic acid levels with respect to the control. The total ascorbic acid level was significantly decreased when EAC cells were developed for 12 (28.44%), 15 (31.56%) and 18 (44.68%) days with respect to their corresponding control. The development of EAC cells for 12 (39.01%), 15 (43.12%) and 18 (45.09%) days also decreased the reduced ascorbic acid level with respect to their corresponding control. No significant change was observed in 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 development of EAC cells for 12, 15 and 18 consecutive days with respect to the control as well as with respect to the only caffeine treated conditions. It is also observed from this Figure that the EAC cell-induced decrease in total ascorbic acid level was restored to the control level when caffeine was administered for 24 (51.47%), 27 (66.35%) and 30 (57.6%) consecutive days, including 12 consecutive days of caffeine treatment prior to EAC cell inoculation. Similarly, the reduced ascorbic acid level in adrenal gland was significantly increased under similar conditions by 23.86%, 38.65% and 41.11% respectively with respect to corresponding EAC cell developing conditions alone.

Discussion

The results of the present study demonstrate 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 1). Caffeine-induced suppression of EAC cell growth may be supported by the works of Theiss and others who in fact, have shown that caffeine suppresses both spontaneous and chemically induced pulmonary adenoma development.

The 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 (Fig. 1a). 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, due to the hyperactivity of the adrenal gland. 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 control (Fig. 1a) suggesting that long-term caffeine treatment may have a negative role over the EAC cell growth. Long-term caffeine consumption which has been found to develop tolerance to caffeine does not appreciably change the corticosterone status.

The hypersecretion of ACTH causes an increase of plasma corticosterone level as well as tumor growth. 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. Thus, the reduced level of adrenal ascorbic acid (both total and dehydro) observed in the present study (Fig. 1b) during the development of EAC cells suggests that EAC cell development may be associated with the enhancement of ACTH secretion followed by elevation of plasma corticosterone level and reduction of adrenal ascorbic acid level (Figs 1a & 1b). 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. 1a & 1b), the continuation of caffeine treatment during EAC cell development suppresses the EAC cell - induced induction of plasma corticosterone level (Fig. 1a) and restores the EAC cell - induced reduction of adrenal ascorbic acid level (Fig. 1b) 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. We have earlier reported that in EAC cells long-term caffeine-induced tolerance does not develop.
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. 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. This phenomenon may be explained by the fact that ACTH-induced induction of intracellular cAMP induces the phosphodiesterase activity, cAMP degrading enzyme, as a result the cAMP level in the system may be down regulated and hence may stimulate the EAC cell 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. Thus inhibition of this enzyme may stimulate / up-regulate the cAMP level in the cellular environment and may reduce the EAC cell growth. Thus, caffeine can inhibit or delay mitosis in many kind of cells, probably due to variations in cAMP concentration that influence DNA synthesis and mitosis. Again, it may also be explained by the fact that caffeine is a potent antioxidant because it is an effective inhibitor of lipid peroxidation. This antioxidant property of caffeine may reduce the EAC cell growth.

In conclusion, 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.

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