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Long-term caffeine-induced inhibition of EAC cell progression in relation to gonadal hormonal status

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Inhibitory action of caffeine (a tri-methylxanthine alkaloid) on progression or pathogenesis of lung, breast and ovarian cancer including Ehrlich ascites carcinoma (EAC) cell development has been reported. This information led the authors to study the effect of long-term administration of caffeine (20mg/kg/day; po for 22-27 consecutive days) on the development of EAC cells in relation to serum gonadal hormones (LH, FSH, 17-OH-β-estradiol (E2) and progesterone) in adult Swiss albino female mice. Measurement of gonadal hormones in serum using RIA showed that (a) long-term caffeine treatment significantly increased LH (except for 27 consecutive days) and decreased FSH (except for 24 and 27 consecutive days) and both E2 and progesterone (except for 22 and 24 consecutive days) levels, (b) development of EAC cell for 10-15 days, significantly increased LH but decreased FSH, E2 and progesterone levels and (c) long-term caffeine consumption during the development of EAC cell (i) restored the EAC cell- or caffeine-induced induction of LH and reduction of FSH level to their normal levels and (ii) withdrew/reduced the EAC cell-induced reduction in only E2 but not progesterone level. These results therefore, suggest that prolonged caffeine exposures may inhibit the development of EAC cell through the reduction or restoration of EAC cell-induced disruption of ovarian hormonal status to their normal status via the modulation of Hypothalamic-Pituitary-Gonadal (HPG) axis.

Keywords: Caffeine, E2, EAC cell, FSH, LH, Progesterone

Caffeine, a tri-methylxanthine plant alkaloid present in coffee, tea and soft drinks has many pharmacologic effects including neuropharmacological and endocrinological effects due to it being similar in structure to endogenous metabolites[1-2]. Caffeine exerts inhibitory effects on the development of spontaneous and inducible cancers[3-8], though there are some contradictory reports regarding this aspect[9-12]. The development of cancer is closely associated with endogenous levels of sex steroids[13,14] and gonadotrophic hormones[15-17] including their receptor population[18-20]. The steroids, which are likely to influence tumorigenesis, are namely, estrogen and progesterone that affect many physiological functions including the reproductive system[21]. The steroid hormones have profound influence on morphogenesis, cell-proliferation, cyto-differentiation and neoplastic transformation of many tissues, which, in turn may affect the pathogenesis or progression of cancer[22,23]. The steroids are synthesized and secreted mainly from ovaries and adrenal glands on the stimulation of Follicle-stimulating hormone (FSH) and lutenizing hormone (LH), which in turn are controlled by the hypothalamic gonadotropin releasing hormone (GnRH) via the involvement of Hypothalamic-Pituitary-Gonadal (HPG) axis[24-26]. The gonadotropins, LH and FSH, are involved in regulation of steroidogenesis, luteinization and maturation of ovarian follicles[27]. The steroidogenic action of LH and FSH is mediated via cAMP dependant protein kinase[28]. The steroidogenic actions of gonadotropins are changed during the different phases of estrus cycle depending on its particular phase[29]. Further, it has been observed that consumption of caffeine alters serum gonadal hormone status depending on the dosage, duration and species[30,31]. Thus, it can be hypothesized that the inhibitory action of caffeine on cancer progression may be a result of the modulation of serum gonadal hormone levels via the HPA-axis/HPG-axis. The present investigation proposes to study the effect of long-term consumption of caffeine, if any, on serum gonadal hormonal status at the estrus

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phase during the development of Ehrlich ascites tumor in female mice.

Materials and Methods

Chemicals—Caffeine was purchased from Fluca Chemica Biochemical (Switzerland). LH, FSH, 17-OH-β-estradiol (E2) and progesterone assay kits and protocols were obtained from M/s DSL Inc., Webster, Texas, USA. All other reagents used in the present study were of analytical grade.

Animals—Adult virgin female mice of Swiss albino strain, aged 50-60 days, weighing between 25-30 g were housed in a 4-6 member group per cage. Animals were maintained in a room at 28°±0.5°C and 85±5% RH under a controlled 12:12 hr L:D cycle. Estrus cycle was monitored daily by vaginal smear as described by Allen *et al*[32] and only the mice exhibiting at least two consecutive 4-day estrus cycles, were considered in the study.

Experimental design—Animals were divided into two groups; Group 1 (control) and group 2 (experimental). Each group was further divided into 3 subgroups. Each sub group contained 3-4 animals which were treated with either caffeine or EAC cells or caffeine in presence of EAC cells according to the protocol given in Fig. 1.

All experiments in the present study conformed to the guidelines on the ethical use of animals and efforts were made to minimize the number of animals and their sufferings.

Collection of material—All animals were sacrificed between 8:00 to 10:00 hr (at the estrus phase) by cervical dislocation. Animals of all caffeine treated groups were sacrificed after 30 min of the last caffeine administration. Whole blood was collected by cardiac puncture in eppendorf and kept for 1 hr at 37°C and then centrifuged at 800 rpm for 10 min at room temperature to separate serum. The serum was stored at -20°C until used.

Assay for hormones—LH, FSH, E2 and progesterone levels in blood were measured by RIA method using commercially available standard kits according to the manufacturer's instructions (Diagnostic System Laboratories Inc. Webster, Texas, USA).

Statistical analysis—Statistical significance between the groups of the experimental values was assessed by Analysis of variance (ANOVA) using Scheffe's multiple comparison test[33].

Results

Treatment of mice with caffeine (20 mg/kg, po) for 22 consecutive days significantly increased LH and decreased FSH without any significant change in E2 and progesterone levels with respect to the corresponding control group (Table 1). However, caffeine treatment for 24 consecutive days significantly increased LH level (except FSH, E2 and progesterone). Prolongation of caffeine treatment up to 27 consecutive days did not show any significant change in LH and FSH levels but significantly decreased E2 and progesterone levels with respect to the corresponding control group.

It is evident from Table 1 that the serum LH level was significantly increased in mice during the development of EAC cell for 10, 12 and 15 days. Unlike LH level, the serum levels of FSH, E2 and progesterone in mice were decreased during the development of EAC cells for 10, 12 (except FSH) and 15 days with respect to their corresponding control.

Further, no significant changes were observed in serum LH and FSH levels in mice, which were pretreated with caffeine for 12 consecutive days and continued for another 10, 12 and 15 consecutive days after the inoculation of EAC cells with respect to the corresponding control group. In contrast, caffeine pretreated (for 12 consecutive days) mice bearing EAC cell which were further treated with caffeine for another (a) 10 consecutive days, significantly decreased E2 and progesterone levels, (b) 12 consecutive days, E2 level was decreased but progesterone level was unchanged and (c) 15 consecutive days, both E2 and progesterone levels were decreased with respect to their corresponding control.

Table 1 also depicts that with respect to EAC cell developing condition alone the treatment with caffeine for 12 consecutive days prior to EAC cell inoculation to mice and continued during the development of EAC cell for (a) 10 days, significantly decreased serum LH and progesterone levels but increased its FSH and E2 levels, (b) 12 days, significantly decreased LH and increased E2 and progesterone levels (except the level of FSH) and (c) 15 days, significantly increased FSH, E2 and progesterone levels (except the level of LH). Table 1 also suggest that with respect to the caffeine treated condition alone caffeine treatment during the development of EAC cells (inoculated following 12

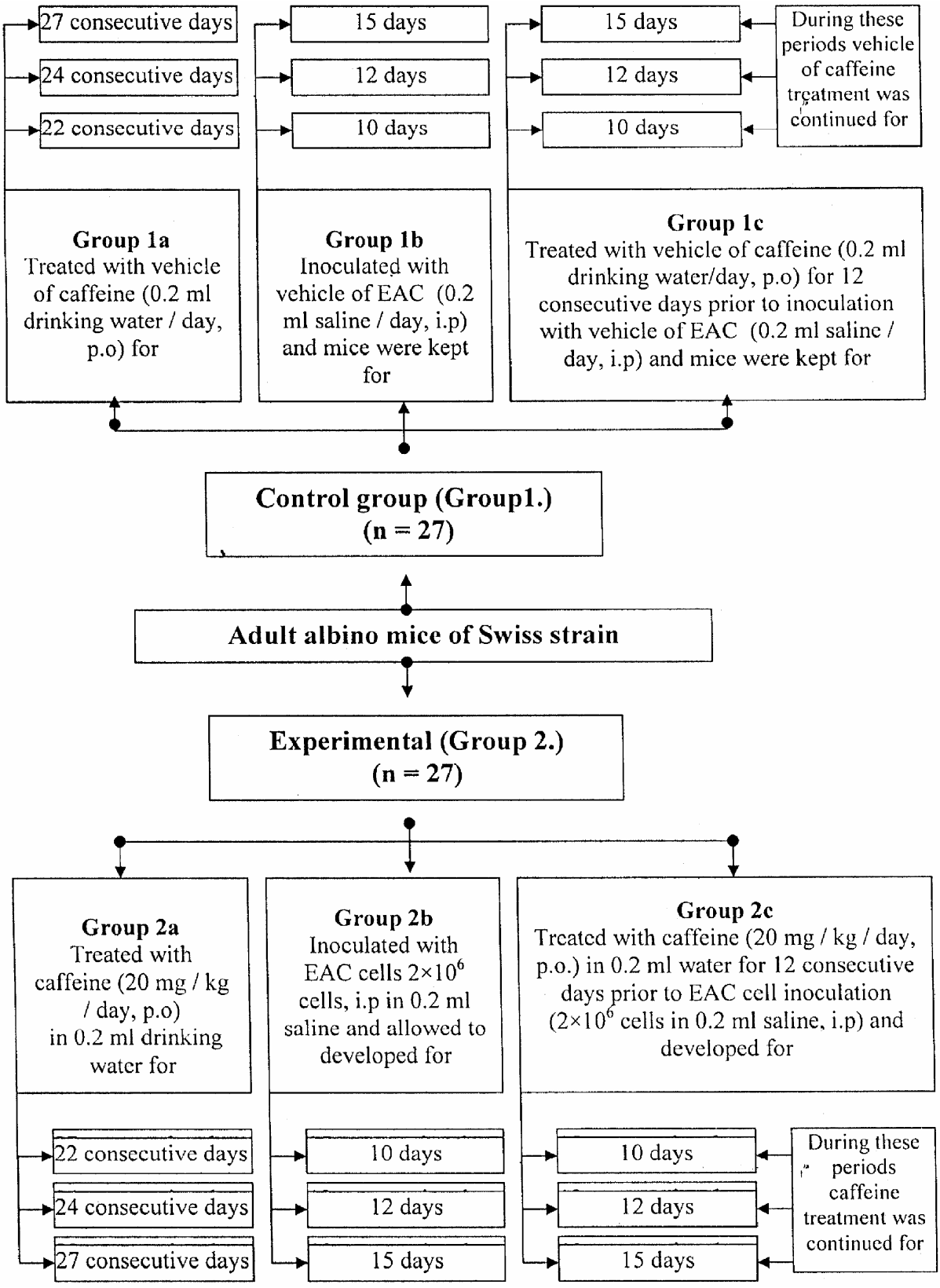

Fig. 1—Protocol for treatment of mice with caffeine or EAC cell or caffeine in presence of EAC cells

Table 1—Effect of long-term consumption of caffeine on EAC cell-induced change in serum LH, FSH, β-estradiol and progesterone levels in adult mice

[Results are expressed as mean ± SEM of 3 separate observations. Figures in parentheses are % increase (+) / % decrease (-) over the control values]

Experimental condition (s)	Duration of caffeine treatment/ Development of EAC cells. (days)	Serum levels of LH (ng/ml) × 10^{-2}		FSH (ng/ml)		β-estradiol (pg/ml)		Progesterone (ng/ml)	
		Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
Caffeine	22	12.33 ± 0.69	17.77 ± 1.07[a] (+ 44.72)	7.61 ± 0.32	4.92 ± 0.13[a] (- 35.35)	23.75 ± 1.52	23.77 ± 0.93 (+ 0.08)	16.75 ± 1.32	15.46 ± 0.63 (- 7.70)
	24	12.27 ± 0.65	23.53 ± 0.67[a] (+ 91.77)	7.60 ± 0.33	7.14 ± 0.18 (- 6.05)	23.40 ± 1.22	19.54 ± 0.25 (- 16.50)	16.74 ± 0.99	13.76 ± 0.36 (- 17.80)
	27	11.77 ± 0.67	13.27 ± 0.67 (+ 12.74)	7.05 ± 0.01	7.43 ± 0.14 (+ 5.39)	24.25 ± 0.39	17.79 ± 0.34[a] (- 26.64)	16.22 ± 1.23	3.15 ± 0.06[a] (- 80.58)
EAC cell development	10	12.33 ± 0.64	21.27 ± 1.23[a] (+ 72.51)	7.58 ± 0.33	4.73 ± 0.72[a] (- 37.60)	23.33 ± 1.24	2.60 ± 0.38[a] (- 88.86)	16.74 ± 0.99	8.45 ± 0.06[a] (- 49.52)
	12	11.87 ± 0.78	18.23 ± 0.87[a] (+ 53.58)	7.58 ± 0.21	6.57 ± 0.36 (- 13.32)	23.53 ± 1.45	6.38 ± 0.67[a] (- 72.89)	16.65 ± 0.97	11.25 ± 0.54[a] (- 32.43)
	15	12.00 ± 0.59	17.53 ± 1.12[a] (+ 46.08)	7.10 ± 0.02	1.34 ± 0.33[a] (- 81.13)	24.93 ± 0.73	5.14 ± 0.34[a] (- 79.38)	16.36 ± 1.25	2.72 ± 1.04[d] (- 83.37)
EAC cell development in presence of caffeine	22 (10)	12.10 ± 0.72	15.53 ± 0.65[d] (+ 28.35)	7.57 ± 0.29	8.07 ± 0.38[b,d] (+ 6.61)	23.80 ± 1.45	17.34 ± 0.51[a,b,d] (- 27.14)	16.73 ± 0.93	4.80 ± 0.41[a,b,d] (- 71.31)
	24 (12)	12.00 ± 0.78	13.53 ± 0.43[b,d] (+ 12.75)	7.63 ± 0.29	7.83 ± 0.12 (+ 2.62)	24.20± 1.15	9.32 ± 0.14[a,b,d] (- 61.49)	16.43 ± 0.88	14.82 ± 0.73[d] (- 9.80)
	27 (15)	11.67 ± 0.67	13.77 ± 0.65 (+ 17.99)	7.18 ± 0.24	9.75 ± 0.46[b,d] (+ 35.79)	24.43 ± 0.88	12.93 ± 1.33[a,c,d] (- 47.07)	16.26 ± 0.96	5.96 ± 0.42[a,b,d] (- 63.35)

Significantly different from the corresponding control group [a]$P<0.01$; caffeine group [b]$P<0.01$, [c]$P<0.05$; cancer group [d]$P<0.01$ using ANOVA

consecutive days of caffeine pretreatment) for (a) 10 consecutive days, significantly increased FSH but decreased E2 and progesterone levels without affecting its LH level, (b) 12 consecutive days, significantly decreased serum LH and E2 levels except FSH and progesterone and (c) 15 consecutive days, significantly decreased E2 and increased FSH along with progesterone levels but not LH in serum.

Discussion

Previous studies[3-8] suggest that caffeine plays an inhibitory role in pathogenesis or progression of ovarian and breast cancer including Ehrlich ascites tumor cell development. It is also known that progression of cancer including ovarian and breast cancer is dependent on the status of gonadal hormones[13-17] as well as their specific receptors[18-20]. Caffeine is known to affect pituitary function[34,35], and modulates the physiological status of ovaries and breast through the modulation of serum gonadal hormones via the interregulation of HPA and HPG axis[30,31]. The objective of the present study was to evaluate the possible mechanism of action of long-term consumption of caffeine, if any, on serum sex hormones particularly the status of LH, FSH, E2 and progesterone at the estrus phase in relation to the Ehrlich ascites carcinoma cell development in female mice.

The hormonal profile of LH, FSH, E2 and progesterone of normal healthy mice as exhibited at different phases of estrus cycle in the present study supports the results reported by Nothmick *et al*[36], Zi-Jian *et al*[37] and Paolo *et al*[38]. The EAC cell progression for 10 days or more, produced a profound endocrine response which may be characterized by significant increase in serum LH level and decrease in serum FSH, E2 and progesterone levels (Table 1). These hormonal changes may be a result of tumor-induced initiation and/prolongation of stress[39-41], which causes an imbalanced in ovarian function possibly via the involvement of HPA-axis and/HPG axis[42,43].

An increase in serum LH following 22/24 consecutive days of caffeine consumption and

decrease in serum FSH and E2 along with its progesterone levels following 22 and 27 consecutive days respectively of caffeine consumption suggest that caffeine-induced respective changes of the threshold levels of these gonadal hormones may disrupt the ovarian function depending on the duration of treatments as well as their normal physiological function[30,31]. Unlike 22 and 24 consecutive days of caffeine treatment, the continuation of the caffeine treatment up to 27 consecutive days, restored the serum LH and FSH levels but not the E2 and progesterone, to normal threshold levels suggesting the development of its tolerance to the HPG axis following 27 consecutive days of long-term treatment. It has been shown[44-47] that caffeine treatment for more than 12 consecutive days develops tolerance to HPA axis. Caffeine-induced decrease of serum progesterone level under this condition needs further investigation to explain the present observations.

Unlike caffeine or EAC cell-induced changes in gonadal hormones as mentioned above, the long-term caffeine consumption during EAC cell development restored the EAC cell- or caffeine-induced (a) induction of serum LH level to the normal at its tolerant as well as non-tolerant conditions and (b) reduction of serum FSH level to the normal at tolerant but not at non-tolerant condition. The EAC cell-induced reduction of serum E2 level under both caffeine tolerant and non-tolerant condition on the other hand, did not restore to its normal value rather it inclined towards the normal (at only caffeine tolerant condition). Unlike those parameters, no restoration of EAC cell- or caffeine-induced reduction of serum progesterone level to its normal level following the treatment of caffeine during the development of EAC cells indicates that caffeine (under its tolerant condition) may reduce the EAC cell-induced disruption of ovarian hormonal level and hence inhibit the progression of EAC cell development by modulating the gonadal hormones possibly through the involvement of HPG axis[42,43] at the estrus phase. The results of the present investigation are in accordance with previous work on neurochemicals where, caffeine under the similar experimental condition have been found to inhibit EAC cell progression through the modulation of central GABAergic activity[7]. The inhibitory role of caffeine in other types of cancer like skin, lung, ovarian and breast has also been reported[3-6]. The present uneven observation of the serum progesterone levels with the pattern of other hormonal levels under the above experimental condition needs further clarifications, which are now in progress in the laboratory.

Though the exact mechanism through which caffeine exerts its inhibitory effect on EAC cell progression is still a question, one of the possible pathway that may be suggested from the present observation is that caffeine may act on HPA/HPG axis and withdraw or reduce the EAC cell-induced disruption of normal gonadal hormonal status through the modulation of HPA-axis/HPG axis to combat the EAC cell-induced progression / changes during the estrus phase. Further works are warranted to understand the mode of involvement and modulation of HPG- and HPA-axis, if any, under the present experimental condition at the level of neuroendocrinology.

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References

1 Kalmar J M & Cafarelli E, Effect of caffeine on neuromuscular functions, *J Appl Physiol*, 87 (1999) 801.
2 Arnaud M J, The pharmacology of caffeine, *Prog Drug Res*, 31 (1987) 273.
3 Nomura T, Inhibitory effect of caffeine on chemical carcinogenesis in mice, *Proc Am Assoc*, 18 (1977) 244.
4 Theiss J C & Shimkin M B, Inhibitory effect of caffeine on spontaneous and urethane-induced lung tumors in strain A mice, *Cancer Res*, 38 (1978) 1757.
5 VanderPloeg L C & Welsch C W, Inhibition by caffeine of ovarian hormone-induced mammary gland tumorigenesis in female GR mice, *Cancer Lett*, 56 (1991) 245.
6 Conney A H, Lu Y P, Lou Y R & Huang M T, Inhibitory effects of tea and caffeine on UV-induced carcinogenesis: Relationship to enhanced apoptosis and decreased tissue fat, *Eur J Can Prev*, 11(Suppl) (2002) S28.
7 Mukhopadhyay S & Poddar M K, Long-term caffeine inhibits Ehrlich ascites carcinoma cell-induced induction of central GABAergic activity, *Neurochem Res*, 25 (2000) 1457.
8 Mukhopadhyay S & Poddar M K, Caffeine inhibits the development of Ehrlich ascites carcinoma cell in female mice, *Indian J Exp Biol*, 39 (2001) 735.
9 Welsch C W, DeHoog J V & O'Connor D H, Influence of caffeine consumption on carcinomatous and normal mammary gland development in mice, *Cancer Res*, 48 (1988) 2078.
10 Welsch C W, Scieszka K M, Senn E R & DeHoog J V, Caffeine (1,3,7-trimethylxanthine), a temperature promoter

of DMBA-induced rat mammary gland carcinogenesis, *Int J Cancer*, 32 (1983) 479.
11 Welsch C W & DeHoog J V, Influence of caffeine consumption on 7, 12-dimethylbenz(a)anthracene-induced mammary gland tumorigenesis in female rats fed a chemically defined diet containing standard and high levels of unsaturated fat, *Cancer Res*, 48 (1988) 2074.
12 Welsch C W, Caffeine and the development of normal and neoplastic mammary gland, *Proc Soc Exp Biol Med*, 207 (1994) 1.
13 Swanson S M, Whitaker L M, Stockard C R, Myers R B, Oelschlager D, Grizzle W F, Juliana M M & Grubbs C J, Hormone levels and mammary epithelial cell proliferation in rats treated with a regimen of estradiol and progesterone that mimics the preventive effect of pregnancy against mammary cancer, *Anticancer Res*, 17 (1997) 4639.
14 Reed M J, Cheng R W, Noel C T, Dudley, H A & James V H, Plasma levels of estrone, estrone sulphate, and estradiol and the percentage of unbound estradiol in post-menopausal women with and without breast disease, *Cancer Res*, 43 (1983) 3940.
15 Gimes G, Szarvas Z & Siklosi G, Endocrine factors in the etiology of endometrium carcinoma, *Neoplasma*, 33 (1986) 393.
16 Bhatavdekar J M, Shah N G, Trivedi S N & Karelia N H, Peptide and steroid hormone levels in pre- and postmenopausal breast carcinoma, *Neoplasma*, 34 (1987) 95.
17 Bhatavdekar J M, Patel D D, Chikhlikar P R, Mehta R H, Vora H H, Kerelia N H, Ghosh N, Shah N G, Suthar T P & Neema J P, Levels of circulating peptide and steroid hormones in men with lung cancer, *Neoplasma*, 41 (1994), 101.
18 Sluyser M, Evers S G & Goeji C C, Sex hormone receptors in mammary tumors of GR mice, *Nature*, 263 (1976) 386.
19 Pujol P, Daures J P, Thezenas S, Guilleux F, Rouanet P & Grenier J, Changing estrogen and progesterone receptor pattern in breast carcinoma during the menustrual cycle and menopause, *Cancer*, 83 (1998) 698.
20 Vihko R, Janne O, Kontula K & Syrjala P, Female sex steroid receptor status in primary and metastatic breast carcinoma and its relationship to serum steroid peptide hormone levels, *Int J Cancer*, 26 (1980) 13.
21 Francesco J D, Bihong Z, Norio T & Sophia Y T, Mechanism of action of estrogen and progesterone, *Ann New York Acad Sci*, 955 (2002) 48.
22 Medina D, Kittrell F S, Shepard A, Contreras A, Resen J M & Lydon J, Hormone dependence in premalignant mammary progression, *Cancer Res*, 63 (2003) 1067.
23 Telang N T, Axelrod D M, Wong G Y, Bradlow H L & Osborne M P, Biotransformation of estradiol by explant culture of human mammary tissue, *Steroids*, 56 (1991) 37.
24 Grossman A, Endocrinology, in *Corticotropin-releasing-hormone: Basic physiology and clinical application*, edited by De Groot L J (Sounders) 1995.
25 Lechan R M, Neuroendocrinology of pituitary hormone regulation, *Endocrinal Metab Clin North Am*, 16 (1987) 475.
26 Baligar P N & Basappa B K, Reproductive toxicity of carbofuran to the female mice, effects on estrus cycle and follicles, *Industrial Health*, 40 (2002) 345.
27 Schwartz N B, Follicle stimulating hormone and leutinizing hormone; a tale of two gonadotropins, *Cancer J Physiol Pharmacol*, 73 (1995) 675.
28 Darbon J M, Ursely J & Leymarie P, Stimulation by LH of cyclic AMP-dependent protein kinase activity in bovine corpus luteum slices, *FEBS Lett*, 63 (1976) 159.
29 Gore-Langton R E & Armstrong D T, Follicular steroidogenesis and its control, in *The physiology of reproduction*, edited by Knobil E & Neil JD (Raven, New York) 1994, 571.
30 Lucero J & Harlow B L, Barbieri R L, Sluss P & Cramer D W, Early follicular phase hormone levels in relation to patterns of alcohol, tobacco, and coffee use, *Fertility Steril*, 76 (2001) 723.
31 Concas A, Porcu P, Sogliano C, Serra M, Purdy R H & Biggio G, Caffeine-induced increase in the brain and plasma concentration of neuroactive steroids in the rat, *Pharmacol Biochem Behav*, 66 (2000) 39.
32 Allen E, The oestrous cycle in the mouse, *Am J Anat*, 30 (1922) 297.
33 Scheffe H, *Analysis of variance* (Willey, USA) 1959.
34 Spindel E, Arnold M, Cusack B & Wurtman R J, Effects of caffeine on anterior pituitary and thyroid function, *J Pharmacol*, 214 (1980) 58.
35 Ezzat A R & El-Gohary Z M, Hormonal and histological effects of chronic caffeine administration on the pituitary-gonadal and pituitary-adrenocortical axis in male rabbits, *Funct Dev Morphol*, 4 (1994) 45.
36 Nothmick W B, Disruption of the tissue inhibitor of metalloproteinase-1 gene results in altered reproductive cyclicity and uterine morphology in reproductive-age female mice, *Biol Reprod*, 63 (2000) 905.
37 Lan Z J, Gu P, Xu X, Jackson K J, DeMavo F J, O'Malley B W & Cooney A J, GCNF-dependent repression of BMP-15 and GDF-9 mediates gamete regulation of female fertility, *EMBO J*, 22 (2003) 4070.
38 Ciana P, Raviscioni M, Mussi P, Vegeto E, Que I, Parker, M G, Lowik C & Maggi A, *In vivo* imaging of transcriptionally active estrogen receptors, *Nature Medicine*, 9 (2003) 82.
39 Weinmam M, Belka C & Plasswilum L, Tumor hypoxia: Impact on biology, prognosis and treatment of solid malignant tumor, *Onkologic*, 27 (2004) 83.
40 Carmeliet P & Jain R K, Angiogenesis in cancer and other disease, *Nature*, 407 (2000) 249.
41 Benhar M, Engelberg D & Levilzki A, ROS, stress-activated kinases and stress signaling in cancer, *EMBO Rep,* 3 (2002) 420.
42 Spicer D V & Pike M C, Breast cancer prevention through modulation of endogenous hormones, *Breast Cancer Res Treat*, 28 (1993) 179.
43 Nilsson U W & Dabrosin C, Estradiol and tamoxin regulate endostain generation via matrix metaloproteinase activity in breast cancer *in vivo*, *Cancer Res*, 66 (2006) 4789.
44 Mukhopadhyay S & Poddar M K, Is GABA involved in the development of caffeine tolerance?, *Neurochem Res*, 23 (1998) 63.
45 Carney J M, Effects of caffeine, theophylline and theobromine on scheduled controlled responding in rats, *Br J Prarmacol*, 75 (1982) 451.
46 Holtzman S G & Finn I B, Tolerance to behavioral effects of caffeine in rats, *Pharmacol Biochem Behav*, 29 (1988) 411.
47 Holtzman S G, Complete, reversible, drug-specific tolerance to stimulation of locomotor activity by caffeine, *Life Sci*, 33 (1983) 779.