

Effect of Depo-Provera on Estrous Cyclicity, Serum Proteins and Lipid Profile in Mice

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Abstract: Depo-Provera[®]; (depot medroxyprogesterone acetate - DMPA) is a contraceptive currently used by women in developed and developing countries. This investigations aimed to monitors its effect on estrous cycle, serum proteins and lipids profile in mice treated with DMPA. Female mice were daily intramuscular injected with DMPA (doses of 0.39 & 0.78 mg) and sacrificed after four or six weeks. Vaginal smears were daily monitored for assessment of estrous cycle phases, sera from both treated and control groups were analyzed for serum proteins and lipid profiles using colorimetric and electrophoretic methods. Results indicated that DMPA induced decrease in the number of estrous cycles, duration of proestrus; estrus and metestrus with concomitant significant ($P < 0.01$) increase in the duration of diestrus. It also, induced biochemical alterations in serum total protein and lipid profiles in the treated compared to control groups. On conclusion, this animal trial shed more light on possible ill-effects of DMPA which may be attributed to hormonal imbalance or toxic effect and this preliminary study indicated a long-term atherogenic role for DMPA.

Key words: Depo provera[®] • Female mice • Estrous cycle • Serum proteins • Serum lipid

INTRODUCTION

Contraception dates back as far as ancient Egypt and Greece. After World War II, the increase in world population was alarming and birth control pill was developed for contraception [1]. Currently, worldwide, more than 90 million women in more than 130 countries depend on injections of long acting depot medroxyprogesterone acetate (DMPA; Depo-Provera[®]) to avoid pregnancy [2]. DMPA is a depot injection containing 150 mg medroxyprogesterone acetate which administered by intramuscular route at a plasma concentration of about 1 ng/mL [3]. Today, nearly more than 12 million women worldwide use injectable, progestin-only formulation over long intervals [4]. Depo Provera, a microcrystalline suspension of medroxyprogesterone acetate (DMPA), is a long-acting, highly effective injectable contraceptive and one of the major means of family planning, yet its use is not without side effects including lipoprotein changes and bleeding irregularities [5 - 7]. After 3 months' use, almost one half of women receiving DMPA injections report amenorrhea, with most of the remainder noting irregular bleeding/spotting. By 1 year of use (4 injections), some three quarters of women using DMPA will experience amenorrhea. Some women view amenorrhea (along with

reduction or elimination of menstrual cramps) as a potential benefit of DMPA use. Adolescent users of DMPA may be more accepting of amenorrhea than adults. Like some adults, many teens actually welcome amenorrhea [8]. Amenorrhea occurs in 55 to 60% of DMPA users at 12 months. Although this is a reversible method of contraception, return of fertility may be delayed for an average of up to nine months [9]. This lack of cardiovascular complications in subjects who receive injections of long-acting DMPA remains to be explained. Lipoprotein changes that may have important effects on cardiovascular health. Serum high density-lipoprotein cholesterol (HDL-C) levels were lowered in women taking DMPA as a twelve-weekly injectable contraceptive for at least one year, as compared to IUD users [10,11]. Levels of albumin, alpha 1-acid glycoprotein, alpha 2-macroglobulin, haptoglobin and IgG were increased following DMPA use for three months by fifty women. While, alpha 1-antitrypsin, transferrin, C3 and C4 levels were reduced [12]. Also, DMPA significantly decreased high density-lipoprotein cholesterol (HDL-C) concentration [13,14]. Serum lipids and lipoproteins were determined during therapeutic amenorrhea induced by lynestrenol and DMPA which associated with smaller atherosclerosis risk due to its weak effect on HDL-C and A1 levels [15]. Lipids known to be

independent risk factors for cardiovascular disease were determined in 24 users of the injectable contraceptive. Both DMPA and norethisterone enanthate (NET), increased LDL total cholesterol decreased high density lipoprotein (HDL) total and free cholesterol which is related to risk of cardiovascular disease in a prospective 12-month study including 18 women [16]. Acute and/or chronic DMPA administration at the level or dose currently-employed for contraception does not induce major abnormalities in serum lipoproteins. While, new acceptors of DMPA were reported with major lipoprotein abnormalities [17] which are unfavorable effects on both lipoprotein metabolism and coagulation profile [18] terms of risk for atherosclerosis [19]. Long-acting, 19-nor-progesterone derivative induce significant increase in IgG levels in healthy Nigerian women in the reproductive age [20]. Also, DMPA, levonorgestrel and gestodene induced marked decrease in the serum protein fractions specially sex hormone binding globulins (SHBG) [21]. The dosage of the progestins appeared to be directly related to the lower concentration of albumin in 4057 current users of progestin including DMPA [22]. DMPA induced alteration of inflammatory status manifested by increase in the concentration of C-reactive protein (CRP) which leads to increase the risk of venous thromboembolism and cardiovascular disease, other oral contraceptive-associated adverse conditions in young women, as well as promoting endothelial dysfunction [23 - 27]. So, the present study was designed to evaluate the effect of DMPA on the cyclicity of the estrous, body weight, both serum proteins and lipid profile.

MATERIALS AND METHODS

Under an approved protocol from the Center for Genetic Engineering, five to seven week-old, mature female CD1 mice (27-35 g) obtained from the Schistosome Biological Supply Program (SBSP) of the Theodor Bilharz Research Institute, Giza, Egypt. These animals were housed in 12h dark/12h light at room temperature and fed standard rodent pellet diet with water *ad libitum*. Six groups of ten animals (n=10) were divided into two sections (3groups/ section) belonging to four and six weeks of injection; one group was assigned as control and two groups were injected with DMPA. Animals were daily intramuscularly injected with DMPA (Depo Provera,[®] Upjohn Kalamazoo, MI) doses (0.39 mg/mouse/day or 0.78 mg/mouse/day). Doses were

calculated to be the mouse equivalent by weight against the human dose of either 150 or 300 mg, using the multiplication factors for interspecies dose conversion [28], or dimethylsulfoxide (DMSO volume control). Body weight gain was calculated as the difference between initial body weight on the 1st day of injection and the final weight taken on before cervical dislocation. Ovary, uterus, liver and kidney were dissected out, freed from adherent tissue and weighed to the nearest milligram. To ensure normalization of data for statistical analysis, organ weights were expressed per 100 g body weight. The phases of estrous cycle were determined by daily observing the vaginal smear in the morning and diestrus index was calculated as number of diestrus days divided on total days of treatment [29]. Also, behavioral and activities of the animals were daily recorded. Animals were sacrificed by cervical dislocation on the end of four or six weeks, 24 h after the final DMPA injection and soon after the last vaginal smear. Heart blood samples were drawn and refrigerated for one hour to clot. Samples were then centrifuged for 5 minutes and the supernatant serum frozen at -20°C for ten days until protein and lipid measurements. Serum total protein, albumin, alpha 1-globulin, alpha 2-globulin, beta-globulin and gamma-globulin fractions were determined by agarose gel electrophoresis (Helena-Laboratories, Texas). The compositions of the electrophoretic solution were constituted from serum protein electrophoretic kit (dye, destaining solutions and gel) (Helena- Laboratories). Serum total protein and albumin were measured by using autoanalyzer HUMASTAR 80 (fully automatic clinical chemistry analyzer, Human Co. GmbH) and its commercial kits. Globulin concentrations were calculated by formulating (Total Protein Concentration = Albumin Concentration + Globulin Concentrations) [30]. The concentration of total lipids was determined according to Zollner and Kirsch [31]. Serum triglycerides were measured by colorimetric methods [32]. Serum total cholesterol was measured using the method of Roschlay and his co-workers [33]. High density lipoproteins-cholesterol (HDL-C) was determined using the method of Jacobs and his co-workers [34]. Cholesterol in very low density lipoprotein (vLDL-C) and low density lipoprotein particles (LDL-C) were calculated using Friedewald's equations [35]. All measurements were performed in triplicate, at least three times. All specimens were kept as aliquots until they could be tested at the same time. The intra-assay error of the method was <3%. A Student's unpaired t-test was employed using the SPSS V.10 software.

RESULTS

Body and Organs Weight: The body and organs (Ovary, uterus, liver and kidney) weight gain were calculated for both control and treated mice with DMPA doses (0.39 or 0.78 mg/mouse/day). The data indicated statistically significant (P <0.01) increase in the body weight and organs weight of the experimental groups when compared with the corresponding control belonging to four and six weeks as shown in Table 1 and these increases were dose and duration dependant.

Estrous Cycle Studies: The control mice exhibited regular estrous cycle and normal duration of each phases of estrous cycle. Treatment with 0.39 or 0.78 mg/mouse/day

caused a significant (P <0.01) decrease in the number of estrous cycle and duration of proestrus, estrus and metestrus with synchronized significant (P <0.01) increase in the duration of diestrus phase. Diestrus index was also increased dose dependently following DMPA injection; these changes were statistically significant (P <0.01) as compared with control as shown in Table 2. Also, the treated mice showed less running activity after DMPA injection and these changes were dose and duration dependant.

Serum Protein Assay: DMPA doses for ten and 15-days induced decrease in the concentration (g/dl) of serum total proteins (maximum ~ -83.10 %). These decreases were statistically highly significant (P ≤ 0.01) when

Table 1: Effect of daily injection of DMPA on body and organs weight of mice

Groups	Body Weight Gain (gm) (Mean ± S.E.)	Relative Weight/100 g Body Weight (Mean ± S.E.)				
		Liver (g)	Kidney (g)	Ovary (mg)	Uterus (mg)	
After 4- Weeks	Control (GI)	2.45 ± 0.32	4.23 ± 0.15	1.26 ± 0.02	26.38 ± 1.34	425.12 ± 5.81
	GII	3.94* ± 0.25	4.96* ± 0.18	1.68* ± 0.11	29.81* ± 1.41	451.23* ± 4.78
	GIII	5.75* ± 0.62	5.82* ± 0.35	2.16* ± 0.21	32.39* ± 1.23	473.19* ± 5.82
After 6- Weeks	Control (GIV)	3.56 ± 0.13	4.78 ± 0.23	1.31 ± 0.07	26.29 ± 1.45	429.22 ± 5.58
	GV	4.89* ± 0.51	5.38* ± 0.43	1.83* ± 0.22	30.13* ± 1.31	462.27* ± 4.81
	GVI	6.19* ± 0.51	6.12* ± 0.31	2.37* ± 0.12	34.68* ± 1.27	483.61* ± 5.83

Results are expressed as means ± SEM. & * = statistically significant

Table 2: Effect of DMPA daily injection on estrous cyclicity of mice

Groups	No. of Cycles (M ± S.E.)	Duration in days (M ± S.E.)					
		Proestrus	Estrus	Metestrus	Diestrus	Diestrus Index	
After 4- Weeks	Control (GI)	5.18 ± 0.31	4.82 ± 0.23	6.76 ± 0.26	5.45 ± 0.28	10.19 ± 0.33	35.71
	GII	4.32* ± 0.64	3.61* ± 0.24	5.96* ± 0.12	4.52* ± 0.22	13.27* ± 0.24	50.00
	GIII	3.12* ± 0.22	2.73* ± 0.26	4.63* ± 0.20	3.49* ± 0.24	14.76* ± 0.28	53.57
After 6- Weeks	Control (GIV)	5.32 ± 0.19	4.65 ± 0.21	6.46 ± 0.22	5.57 ± 0.24	10.89 ± 0.32	39.28
	GV	3.34* ± 0.33	3.79* ± 0.24	4.71* ± 0.28	4.32* ± 0.23	13.85* ± 0.23	52.00
	GVI	1.38* ± 0.28	1.97* ± 0.29	3.77* ± 0.31	3.29* ± 0.26	14.95* ± 0.39	61.34

Results are expressed as means ± SEM. & * = Statistically significant

Table 3: Concentrations of serum total proteins, albumin, globulin (mg/dl) and albumin/globulin ratio of treated female mice with DMPA doses for 4- and 6-weeks

Groups	After Four Weeks (Means ± SEM)			After Six Weeks (Means ± SEM)		
	Control (GI)	GII	GIII	Control (GIV)	GV	GVI
Total Protein	5.29 ± 0.13	4.62* ± 0.15	3.15* ± 0.12	5.38 ± 0.14	4.54* ± 0.23	2.98* ± 0.18
Albumin	1.91 ± 0.06	1.19* ± 0.05	0.94* ± 0.03	1.87 ± 0.09	1.60* ± 0.02	0.85* ± 0.03
Globulin	3.36 ± 0.30	3.64* ± 0.11	4.19* ± 0.07	3.33 ± 0.16	4.24* ± 0.08	4.59* ± 0.03
A/G ratio	56.84	32.69	22.43	56.15	37.73	18.51

Results are expressed as means ± SEM. & * = Statistically significant

Table 4: Effect of DMPA on serum globulin concentrations (mg/dl) of mice

Groups	After Four Weeks			After Six Weeks		
	Control	GII	GIII	Control	GV	GVI
Alpha1	2.17 ± 0.23	2.66* ± 0.13	2.92* ± 0.16	2.21 ± 0.14	2.62* ± 0.11	3.42* ± 0.02
Alpha2	2.29 ± 0.13	2.67* ± 0.11	3.12* ± 0.14	2.24 ± 0.14	2.86* ± 0.17	3.32* ± 0.20
Beta	0.86 ± 0.22	1.06* ± 0.12	1.46* ± 0.23	0.84 ± 0.32	1.18* ± 0.23	1.63* ± 0.31
Gamma	1.49 ± 0.13	1.57* ± 0.16	1.75* ± 0.18	1.48 ± 0.15	1.77* ± 0.21	2.23* ± 0.14

Results are expressed as means ± SEM. & * = statistically significant

Table 5: Effect of DMPA on serum lipid profile of mice

Groups	After Four Weeks			After Six Weeks		
	GI	GII	GIII	GIV	GV	GVI
TL	279.41 ± 3.19	288.13 ± 4.10	310.15 ± 2.52	281.10 ± 3.12	294.14 ± 2.73	343.20 ± 3.17
TG	45.51 ± 1.74	56.28* ± 3.70	62.34* ± 1.37	46.17 ± 1.51	58.29* ± 1.46	67.35* ± 1.92
TC	115.62 ± 2.34	128.40* ± 3.31	139.76* ± 2.25	118.20 ± 2.13	144.10* ± 1.81	158.11 ± 2.82
HDL-C	51.90 ± 1.32	45.34* ± 1.36	36.80* ± 1.36	52.32 ± 1.24	41.38* ± 1.98	34.10* ± 2.80
LDL-C	53.20 ± 1.53	62.41* ± 1.83	74.64* ± 1.43	52.63 ± 1.61	71.49* ± 1.26	84.14* ± 1.61
vLDL-C	7.64 ± 0.21	11.61* ± 1.9	13.32* ± 1.40	8.41 ± 1.33	12.62* ± 1.24	14.82* ± 1.82

Data expressed as: mean ± standard error , *= significant, TL= total lipids, TG = triglycerides, TC = total cholesterol, (vLDL-C) = Very low density lipoprotein Cholesterol, (HDL-C)= High density lipoprotein Cholesterol, (LDL-C)= Low density lipoprotein Cholesterol

compared with the control group (Table 3). Also, DMPA induced decrease in the concentrations of albumin; globulin and the ratio albumin to globulins were significantly (P <0.01) decreased. Moreover, both α1-globulin and particularly α2-globulin, Beta-globulin and gamma-globulin concentrations decreased in all groups (Table 4). These alterations were dose and duration dependant.

Serum Lipid Profile Assay: DMPA induced several alterations in lipid profile in the injected mice which were dose and duration dependant and manifested by increase in the concentration of total lipids, triglycerides, total cholesterol, low density lipoprotein- cholesterol (LDL-C) and dose-related increase in very low density lipoprotein-cholesterol (vLDL-C). While, it induced decrease in HDL-C as shown in Table (5); all these alterations were statistically significant (P <0.01).

DISCUSSION

Human equivalent doses of 150 and 300 mg DMPA were administered to intact adult virgin female mice and the estrous cyclicity, serum proteins and lipids were observed for four or six weeks. Changes that are consistent with those in women taking similar doses were observed. These effects included body and organs

weight gain, alterations in estrous cycle, decrease in the concentration of total proteins, albumin, globulin (mg/dl) and albumin/globulin ratio as well as α1, α2, β and γ-globulin concentrations. Moreover, decreases in HDL and increases of total lipids, triglycerides, cholesterol and LDL; which were in accordance with several published articles. Many studies were carried out concerning the deleterious health consequences of overweight and obesity during DMPA administration and some women attribute their weight gain to such use [36 - 45]. Although there have been anecdotal reports that most hormonal contraceptives are associated with little or no effect on body weight [27]. Some studies have failed to find that DMPA is associated with significant weight gain [46]. In experimental animals, DMPA was found to induce weight gain in female rats (2.7 or 5.4 mg/rat for ten and fifteen days) [47]. Weight gain during DMPA use attributed to its anabolic effects and fluid retention [48, 49] which could induce modifications on the hypothalamic appetite control center associated with the use of DMPA [46] or due to fat deposition, higher appetite and dietary ingestion [51]. In this study DMPA induced estrous cycle alterations manifested by reduction of duration of proestrous, estrus and metestrus as well as elongation of diestrus duration. The normal menstrual cycle is guided by the steroid hormones estrogen and progesterone which act primarily through

and subsequently regulate expression of their specific receptors. The different methods of delivering contraceptive progestin to the uterus result in various expression of steroid receptors in the endometrium, which may contribute to the bleeding disturbances [41]. Hypoalbuminemia was reported in subjects treated with progestins and attributed to the direct effect of progestins on the metabolism of proteins [22]. Reduction of the concentration of Alpha-globulins resulted by immune diseases associated with pregnancy and elevation of progesterone [52, 53]. The binding affinity of both ethinyloestradiol (EE) and DMPA to plasma proteins reached more than 90%, binding occurred mainly to globulins, sex hormone binding globulins (SHBG) and sometimes to albumin [54,55]. Iron-deficiency anemia induced among the users of the contraceptives and it associated with menstrual irregularities. Hormonal contraceptive exert their effect via reduction of hemoglobin and ferritin levels as published by WHO in 1998 [56]. The present study showed decrease in β -globulins level in all treated mice; which usually reduced during pregnancy and oral contraceptives, chronic liver disease, during both acute inflammations and renal disease [57, 58]. Female sex hormones exert its effect on IgG (γ -globulin) which may contribute to the predominance of certain immunological disorders in females. Taken together, the responses of mice seem good models for pharmacological evaluation of possible effects of DMPA in women [59]. Similar results were reported [60] when the female rats were injected with DMPA. Serum lipid profile alterations recorded in the present study are similar to those previously reported in mice and humans [16, 61- 64] and imply that the model may be useful in studying the effects of DMPA-induced lipid changes on the vascular system. Thus far, no such studies have been reported, despite the discrepancy between lipid changes and atherosclerosis in reproductive age women. While, there is an apparent relationship between the biochemical effects of DMPA treatment of mice and women, this study did not explore the effects on atherosclerosis. This inflammatory disease, with lipoproteins, vascular endothelial cells, monocytes, macrophages, smooth muscle cells, activated T lymphocytes and platelets all interacting through adhesion molecules, cytokines, chemokines and prothrombotic factor is a more complex issue and should be tested directly before such a relationship can be assessed. Under most circumstances, atherosclerosis is a chronic disease that becomes clinically apparent after the cessation of ovarian follicle development in aging females [65]. High density lipoprotein-cholesterol is more

closely related to cardiovascular disease in women than low density lipoprotein-cholesterol [34] and is the best predictor of coronary arteries disease risk in women. Furthermore, these results were obtained in normal healthy adult female mice. It is not possible to assess what would have been the result of treatment of abnormal animals or those receiving both estrogen and progestin, as in the case of other contraceptives or menopausal hormone treatment [66,67], or have abnormal medical conditions [61,68]. In conclusion; this animal trial shed more light on possible ill-effects of DMPA which may be attributed to hormonal imbalance or toxic effect and this preliminary study indicated a long-term atherogenic role for DMPA.

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