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Metabolic effects of 20-OH-Ecdysone in ovariectomized rats

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ABSTRACT

Postmenopausal women develop often obesity which may be prevented by 20-OH-Ecdysone (Ecd). This was investigated in ovariectomized (ovx) rats. They were orally treated with 3 doses of Ecd (18, 56 or 116 mg/day/animal). Positive controls received 159 μg estradiol (E2). Quantitative computer tomography at the level of the abdomen and the metaphysis of the tibia allowed estimation of surface, fat depots and muscles. The highest dose of Ecd resulted in serum concentrations of 0.4×10^{-6} M. Serum E2 concentrations in the positive controls were 73.3 ± 24.41 pg/ml. E2 but not Ecd stimulated uterine weights. Under Ecd ovx animals gained less fat but had more muscle mass. Serum TSH, T4 and T3 levels remained unaffected while E2 treatment increases T4 but decreases T3 levels. Ecd at the lowest dose lowered serum LDL and did not result in increased serum triglycerides, an effect seen in the E2 treated rats. At the Ecd highest dose serum HDL was higher than in the controls.

In conclusion Ecd has beneficial effects on fat and muscle tissue and may be able to prevent the metabolic syndrome and sarcopenia by a non-estrogenic mechanism.

1. Introduction

Chronic obesity reaches epidemic dimensions and may result in the metabolic syndrome of which the end stage is type II diabetes, hypertension and arteriosclerosis with all consequent following diseases such as heart attacks and strokes [1–4]. According to WHO 1 billion people are overweight in the world. Thereof 300 million people are morbidly obese. For several reasons particularly aged people are prone to put on weight [5–7] and therefore the metabolic syndrome develops frequently in the postmenopause [8,9]. In addition aged persons often develop a loss of skeletal muscle (sarcopenia) which decreases their mobility [10,11]. It is long known that ovariectomized (ovx) rats develop obesity [6,12] and that hormone replacement therapy (HRT) of postmenopausal women or estrogen treatment of ovx rats influence the development of the metabolic syndrome positively particularly in conjunction with increased bodily exercise and food restriction [6,7]. In the past few years however, HRT was less frequently practiced because of a number of adverse side effects such as a slight increase in the incidence of mammary cancer and of arteriosclerosis which resulted in increased cases of heart attacks and strokes [13]. Hence, other means to prevent the metabolic syndrome and sarcopenia are currently investigated.

Ecdysteroids particularly 20-OH-Ecdysone (β-Ecdysone = Ecd) are known to be produced by arthropods to initiate metamorphosis, the so-called molting process [14] and a number of plants produce ecdysteroids which protect them from herbivory [15–17].

In mammals including the human Ecd is known to stimulate muscular growth [15–17]. We observed recently that the administration of Ecd has antiosteoporotic effects in ovx rats [18]. The ovx induced increase of body weight is due to fat accumulation and can be prevented by E2 treatment which has antilipotropic/lipolytic effects and increases mobility and thereby energy expenditure [5,6]. In our previous experiments we observed that the Ecd treatment did not result in lower body weight in comparison to the ovx control rats. This promoted us to speculate that more muscles were formed on the account of less fat accumulation. Therefore we determined in the present study the amount of fat depots by quantitative computer tomography (qCT). Utilizing this qCT method we have recently described a method to quantify intraabdominal fat depots as well as small fat depots in the lower hind leg which we called paratibial fat depot [19]. These fat depots are sensitively regulated by estrogens and increase dramatically in size following ovx [19–22]. The qCT plane of the lower hind leg details primarily 3 compartments: the surface of bones, of fat depots and muscles. In the present contribution we propose therefore that quantification of fat and of bone surfaces allows calculation of lower hind leg muscle surface and this is also determined in the present study. If in Ecd treated ovx rats fat accumulation is prevented and muscle accumu-

Abbreviations: Ecd, 20-OH-Ecdysone; ovx, ovariectomized; E2, estradiol; HRT, hormone replacement therapy; qCT, quantitative computer tomography; OECD, Organisation of Economic Cooperation and Development; sf, soy-free; TSH, thyroid stimulating hormone; T4, thyroxin; T3, triiodothyronine; SEM, standard errors of means; L4/L5, lumbar vertebral body 4/5; RXR, retinoid x-receptor.

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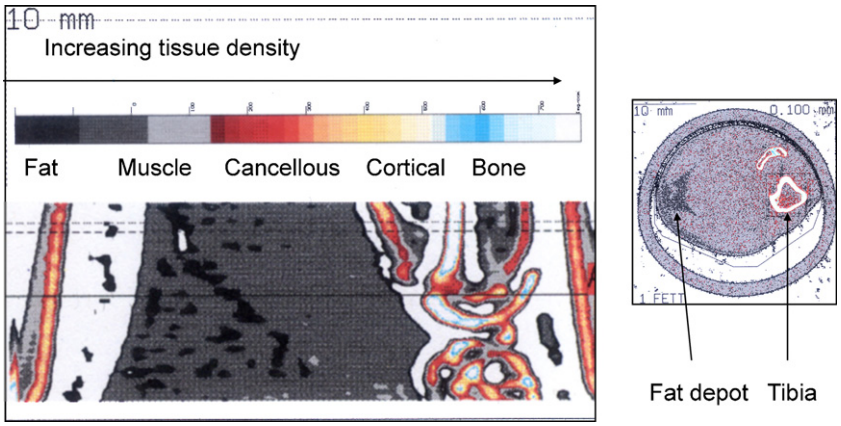

Fig. 1. Scout view and qCT slice of tibial fat depot.

lation increased serum leptin levels may also be affected by Ecd. Leptin is a product of adipocytes and is therefore high in ovx obese and low in intact and E2 treated ovx animals [6,23]. In addition serum cholesterol, LDL and HDL as well as triglycerides, TSH, T3 and T4 levels were measured as additional metabolic parameters.

If Ecd exerts similar beneficial effects as E2 but without the adverse effects of estrogens an estrogenic action of the ecdysteroid in the uterus must be excluded. The Organisation of Economic Cooperation and Development (OECD) recommends testing of estrogenicity by the so-called *uterotropy-assay* which utilizes the growth promoting effects of estrogens in uteri of ovx rats [24,25]. Therefore, in the present study we investigated the effects of 3 orally administered doses of Ecd given over a period of 3 months on the above mentioned parameters and on uterine weights and compared them with those in ovx and E2 treated ovx rats. In essence we hope that Ecd may be used as a means to prevent obesity, arteriosclerosis and sarcopenia.

2. Materials and methods

Female Sprague Dawley rats ($n = 60$) were used for the present experiments. Allowance to perform these experiments was obtained from the Bezirksregierung Braunschweig (permission No. Az.G 82.06). The test substance was 20-OH-Ecdysone (=β-Ecdysone = Ecd) provided by Changzhou Dahua Imp. and Export (Group) Corp. Ltd. Changzhou, Jiangsu, China, 97.2% purity. For control purposes estradiol-17beta (E2)-benzoate (Order no. E-9000, Sigma–Aldrich, St. Louis, MO, USA) was tested.

Three months old Sprague Dawley rats (Winkelmann, Borken, Germany) weighing 250 ± 10 g were adjusted to our animal facilities (6 animals/cage; 12 animals/group; light phase 06.00 a.m. to 06.00 p.m., relative humidity 55%) and were kept on soy-free, pelleted food (sf food) (V 1355 R-Z, 10 mm, poor phytoestrogens, ssniff, Borken, Germany) in which isocaloric protein supplementation was secured by added potato proteins. After 1 week of adjustment animals were anaesthetized with isoflurane (Forene, Abbott-AG, Baar, Switzerland), weighed and subjected to quantitative computer tomography (qCT with the XCT Research SA, Stratec Medizintechnik, Pforzheim, Germany) for the determination of fat depots in the abdomen of the levels of L4/L5 and of a small fat depot in the lower hind leg which we named paratibial fat depot. The scanner was positioned at the level of L4/L5 and 3 tomographic slices were taken. For evaluation of the fat depot in the lower hind leg the scanner was positioned at the epiphysis of tibia and a coronal computed radiograph (Fig. 1) was carried out. The scout view was used to position the scanner at the site of measurement and 3 tomographic slices at a distance of 3.75 or 4.25 or 15 mm distal of the reference line were used for the determination of fat depots.

In these planes the amount of fat and bone tissue was calculated and all values below 40 mg/cm^3 were considered to be fat, between 40 and 99 mg/cm^3 muscle and values between 280 and 400 mg/cm^3 cancellous bone. The qCT plane of the tibia at the level of the upper metaphysis is shown in Fig. 1. In this qCT plane the densities of the different tissues are shown in different grey tones. Computer assisted perimetry of the surface of the fat depot allows calculation of fat surface in mm^2. Similarly the surface of the tibia and fibula can be calculated. The total surface of the CT plane minus surface of fat and bone tissue gives an estimate of the muscle surface in this qCT plane. It is known that ovx animals continue to grow and to develop obesity while intact or E2 treated animals grow less and remain slim [19]. As a consequence the absolute values of muscle tissue surface in the qCT plane are misleading and therefore these surfaces are given in absolute values/100 g body weight while changes in fat tissue are given in % of the pre-treatment values.

After this initial CT-scan rats were ovariectomized (ovx) under the same anaesthesia. After ovx control animals were maintained on soy-free potato protein enriched food, the treated ovx animals were placed on Ecd and E2 containing food for 3 months ($n = 12$/group). On the basis of the twice weekly measurement of food consumption per cage divided by the number of animals per cage the food intake of each rat could be estimated. The type of food, Ecd content of the food as well as average food intake, final body weights (BWs) and the estimated amount of daily uptake of the test substances are detailed in Table 1. After the treatment period

Table 1
Food intake and calculated daily uptake of the test substances.

	Dose	Food intake in ovx rats g/animal/day	Substance intake mg/animal/day	Final body weight in g
ovx, sf	V 1355 ssniff	17.32	–	324.7 ± 21.47
ovx + E2	10 mg/kg food	15.92	0.159	274.5 ± 23.69*
ovx + Ecdysone	1 g/kg food	18.02	18.02	324.4 ± 30.46
ovx + Ecdysone	3 g/kg food	18.86	56.58	332.0 ± 21.09
ovx + Ecdysone	6 g/kg food	19.3	115.8	344.5 ± 10.86

* $P < 0.05$ vs ovx, sf.

of 3 months animals were again subjected to qCT measurement of the fat depots in the abdomen and lower hind leg. After fastening overnight animals were decapitated and blood samples collected from the trunk. The uteri and various organs were removed, cleaned from adherent tissue, weighed and stored at −70 °C.

After termination of the experiments the blood samples were centrifuged (3000 rpm for 10 min) and the serum stored at −20 °C for further analysis. Thyroid stimulating hormone (TSH) was measured by specific RIA supplied by the National Hormone and Pituitary Program of the NIH (Dr. A.F. Parlow, Harbor General Hospital, Torrance, CA, USA), as described previously thyroxin (T4), triiodothyronine (T3), leptin and serum E2 were assayed with commercially available kits (Beckman Coulter, Sinsheim, Germany). Serum cholesterol, HDL, LDL and triglycerides were measured with Hitachi 902 (Roche Diagnostics, Mannheim, Germany) using a commercial kit.

To recover Ecd from serum, enzymatic hydrolysis of potential metabolites was performed before serum extraction. A volume of 500 μl serum was extended with 500 μl NH acetate buffer (pH 5.0) containing 1 mg β-glucuronidase (Helix Pomatia β-Glucuronidase Type H1; Sigma, Taufkirchen) and incubated overnight at 37 °C. The Strata X solid-phase extraction method (8B-S100-UBJ, Phenomenex, Aschaffenburg) with a polymeric sorbent was used according to the instructions of the manufacturer. The eluted volume was evaporated to dryness and reconstituted with 100 μl EtOH. For HPLC a volume of 20 μl was chromatographed over a NC 2504.6 mm Hypersil-ODS 5.0 μm column (Bischoff, Leonberg, Germany). Ecd was detected at 254 nm.

2.1. Statistical evaluation

Data were expressed as means ± standard errors of the means (SEM). Significant differences between the control and treatment groups were analysed by one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons (Prism™, Graph Pad, San Diego, USA). P values < 0.05 were considered statistically significant.

3. Results

Table 1 details the daily food intake and the thereby calculated daily uptake of the test substances as well as the final body weights of the animals. Food intake and final body weights were lowest in the E2 treated animals whereas they did not vary significantly in the Ecd treated in comparison to control animals. Serum E2 concentrations in the E2 treated animals were 73.3 ± 24.41 pg/ml, whereas serum E2 concentrations in the control and Ecd treated animals were undetectable, i.e. below 10 pg/ml. Serum Ecd concentrations in the animals exposed to the highest Ecd concentrations in the food were 0.4×10^{-6} M concentrations in those treated with lower amounts were below the detection limit of the assay system.

Uterine weights in the E2 treated animals were high but low in the controls as well as in the Ecd treated animals regardless whether treated with low or high amounts of the ecdysteroid (Fig. 2). The changes of fat accumulation of abdominal fat tissue as determined by qCT at the level of L4/L5 are shown in Fig. 3a. Since the E2 treated animals remained much lighter and smaller muscle area is given in mm^2/100 g body weight (BW). Largest fat depots were present in the ovx animals while significantly less fat tissue covered the abdominal cavity in the E2 treated animals. Also the Ecd treatment at all doses tested had a slight though significantly smaller effect than E2 in reducing the total abdominal fat mass. A similar observation was made in the paratibial fat depot and no dose–response effect was observed (Fig. 3b); Fig. 4 details

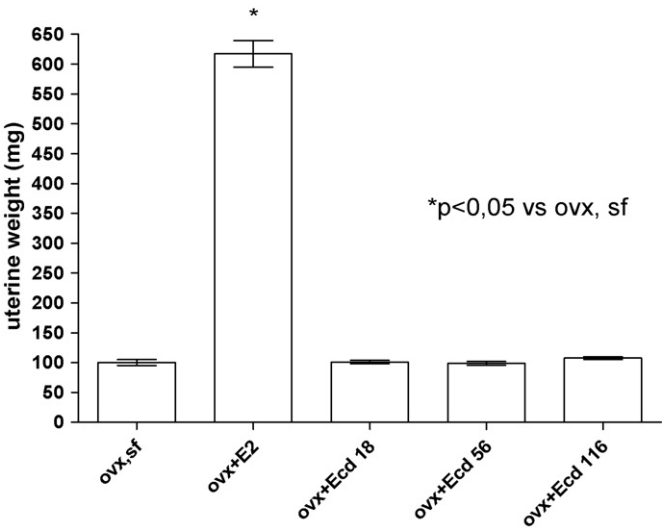

Fig. 2. In comparison to animals kept under soy-free [13] food uterine weights of ovariectomized (ovx) were largely increased in the E2 but not in the Ecd treated animals which had similarly low values as the ovx controls.

high serum leptin values in the ovx controls which were largely reduced in the E2 and partially (but significantly) by all 3 doses of Ecd.

In the upper part of the lower hind leg the total surface of the qCT plane consists primarily of bone, fat and muscle tissue. Subtraction of bone and fat surface gives therefore an estimate of the muscle surface in the qCT plane of the lower hind leg and these values are shown in Fig. 5. Highest relative muscle mass, given per 100 g/BW was present in the E2 treated animals while the ovx animals had lowest muscle mass. The Ecd treated animals had significantly more relative muscle mass than the controls but less than the E2 treated rats.

Serum cholesterol, HDL, LDL and triglyceride levels are shown in Fig. 6a–d. Lowest cholesterol levels were present in the serum of the E2 treated animals (Fig. 6a) and this was primarily due to largely reduced HDL levels (Fig. 6b). The Ecd high treated animals had slightly higher HDL levels (Fig. 6b) and at the low dose reduced LDL values (Fig. 6c) in comparison to the negative control. Most obvious differences in the effects of Ecd in comparison to E2 were found on serum triglyceride levels which were significantly increased in the E2 but unchanged in the Ecd treated animals (Fig. 6a).

Ecd treatment exerted no significant effects on TSH, T4 or T3 while administration of E2 resulted in unaffected TSH, increased T4 and decreased T3 serum levels.

4. Discussion

In a previous publication we showed that the molting hormones of arthropods β-Ecdysone (Ecd) do not bind to estrogen receptors [18] and in the present experiments we demonstrate that it is devoid of uterotrophic effects. The effects of E2 in the treatment of the positive control animals were as expected: it stimulated uterine weight which is the OECD recommended parameter to determine estrogenicity. Hence, according to the OECD criteria Ecd is devoid of estrogenic activity [24,25].

In mammals Ecd is known to have muscle anabolic effects [15–17]. It is therefore frequently taken in g-quantities by bodybuilders to increase their muscle mass. These muscle mass improving properties were confirmed in the present experiments. In the qCT plane of the lower hind leg subtraction of bone and fat surface from the total CT plane surface allowed an estimation of the

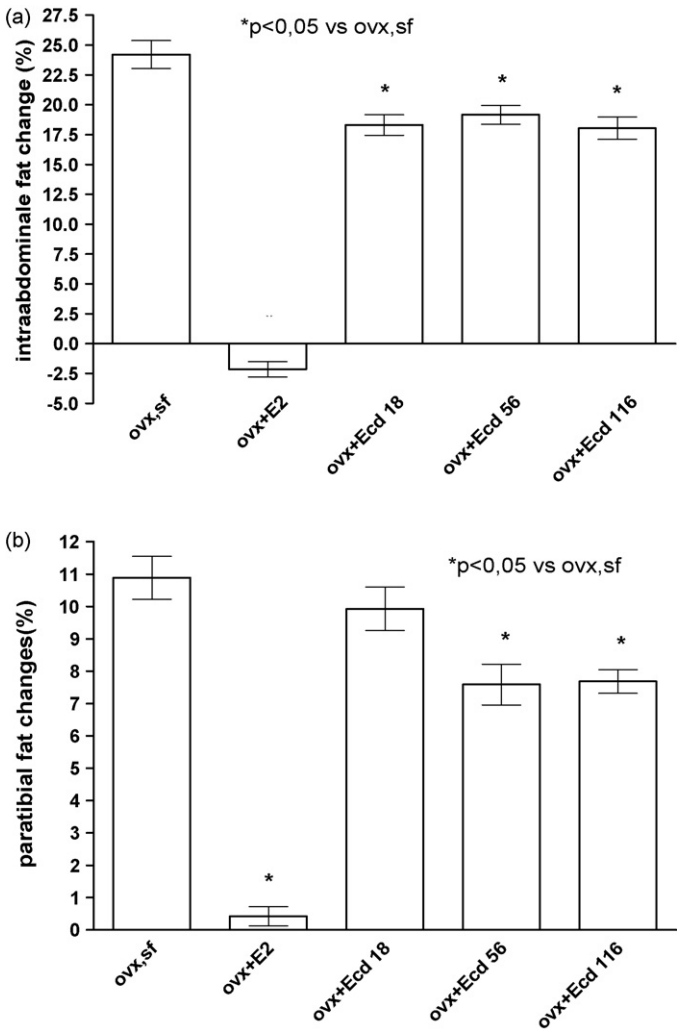

Fig. 3. The amount of intraabdominal fat in ovx rats kept under soy-free [13] food increased markedly and was slightly reduced in Ecd fed animals and at intermediate values in comparison to preovx values (a). Similarly, the increase in paratibial fat tissue was largest in control animals and smallest on the E2 fed. Ecd at the intermediate and highest dose reduced the accumulation of fat tissue (b). The abscissa is the reference line of the sizes of the fat depots prior to ovx. *<0.05 vs animals kept under soy-free [13] food.

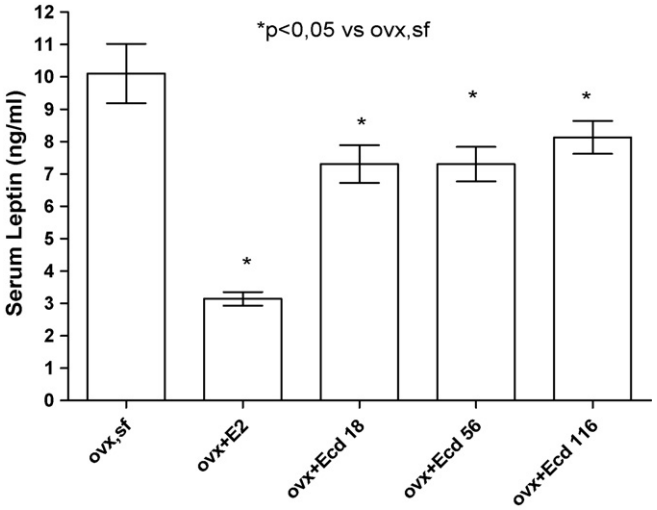

Fig. 4. Serum leptin concentrations were significantly lower in the E2 and the Ecd treated rats. *<0.05 vs animals kept under soy-free [13] food.

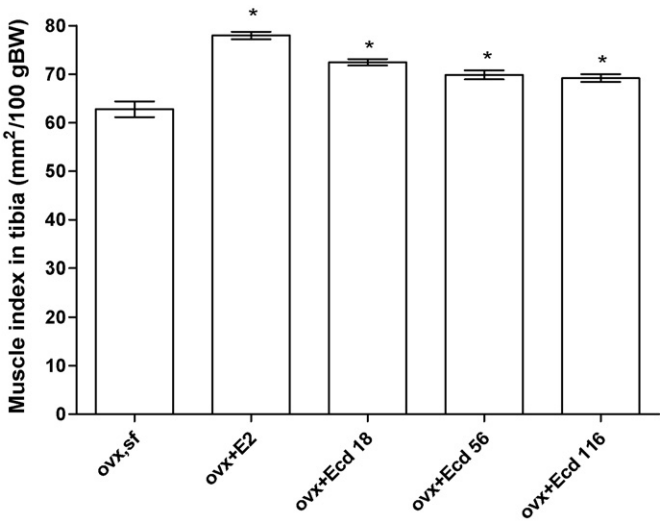

Fig. 5. Muscle index in the qCT plane of the upper tibia was highest in the E2, slightly lower in the Ecd treated animals and lowest in the ovx soy-free [13] fed controls.

total muscle mass and this was highest in the E2 treated animals, at intermediate values in the Ecd and lowest in the ovx controls. In contrast to the stimulatory effects on muscle mass a reduction of both, abdominal and paratibial fat depots was observed. As a consequence of the low load with fat tissue the adipocyte hormone leptin was also reduced. In our earlier experiments [18] we observed no differences in the body weight of ovx and ovx Ecd treated animals and this was confirmed in the present study. This observation that the final body weight after a 3 months feeding period with Ecd resulted in similarly high final body weights as that of the ovx animals was surprising but can be explained on the basis of the presented data: the ovx animals gained weight due to the accumulation of fat tissue while the Ecd treated animals fat tissue did not accumulate but more muscle mass was built up such that the net effect on body weight was similar in both groups. In the E2 treated animals also no fat tissue was built up. However, also the total muscle mass was not increased and this explains the significantly lower body weight in the E2 treated animals. Such fat reducing effects of Ecd were not published hitherto and may point to putatively beneficial effects on the metabolic syndrome.

The high serum cholesterol and accumulation of fat tissue following ovx make the rat a particularly suitable model to study the metabolic syndrome. E2 is known to prevent fat tissue accumulation and to reduce serum cholesterol levels in many castrated mammals and in postmenopausal women [3,4,20]. This was confirmed in the present study to occur also in ovx rats. It was also shown previously that E2 increases serum triglycerides and such effects were also described to occur in postmenopausal women [8,9] practicing HRT. Triglycerides and LDL are the “bad” lipids. Therefore, the observation that all 3 doses of Ecd did not result in increased triglycerides and the modest reduction of LDL in the animals under the low dose of Ecd may be an indication that the Ecd treatment may prevent arteriosclerosis. In addition the stimulation of HDL (the “good” lipoproteins) by the high dose of Ecd would also be beneficial if occurring in postmenopausal women.

A reliable determination of Ecd concentrations in serum was only possible in the high dosed animals in which the Ecd was present in 0.4×10^{-6} M concentrations. Consequently the concentrations were most likely much lower (probably by a factor of 5–6) in the low dosed animals in which significant effects on fat and muscle mass as well as on cholesterol and LDL were seen.

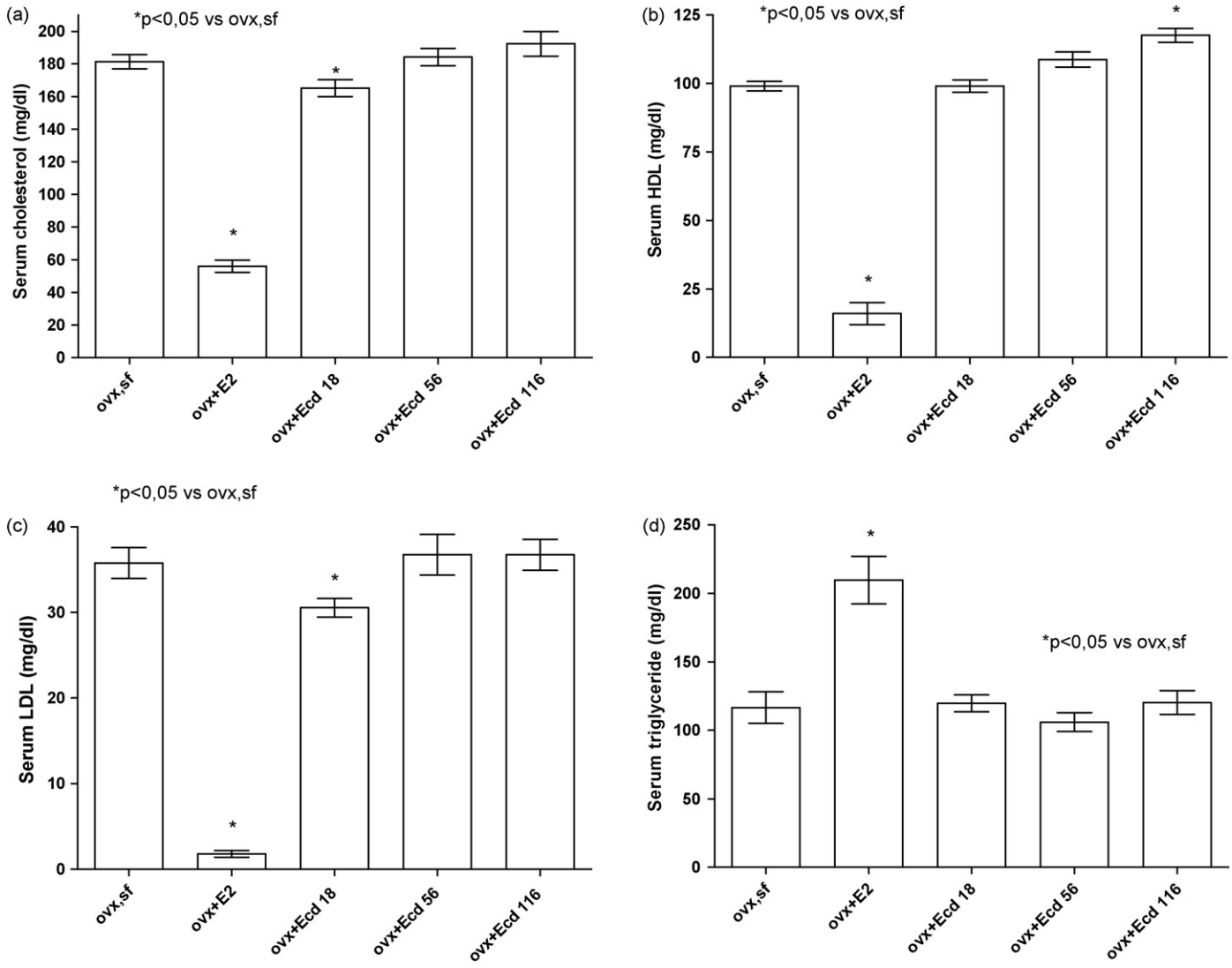

Fig. 6. Cholesterol (a) concentrations were high in ovx, much lower in E2 treated animals and slightly lower in the animals treated with the lowest Ecd dose. The reduction of cholesterol by E2 was due to largely reduced serum HDL (b) and LDL (c). In contrast, in the Ecd treated animals serum HDL was slightly increased which was significant for the highest dose whereas LDL was slightly decreased in animals under the lowest Ecd dose resulting in similar serum cholesterol (a) values as measured in the ovx control animals. Serum triglycerides were increased in the E2 and unaffected in the Ecd treated animals (d).

Treatment with Ecd had no effect in the hypothalamo-pituitary–thyroid axis. Serum TSH, T3 and T4 levels were in the range of the ovx controls. The stimulating or inhibiting effects of E2 on T4 or T3 respectively, deserve a brief discussion. In previous experiments we observed an inhibitory effect of E2 on liver deiodinase activity [26] and this may explain the increased T4 and decreased T3 levels. An alternative or additional explanation is the fact that a number of substances including E2 alter thyroid hormone binding proteins that interfere with the elimination of thyroid hormones or with their access into specific tissue [26].

Ecd receptors are not present in organisms of mammals, the receptor(s) mediating the effects of Ecd is totally unknown [15–17]. There is some evidence that ecdysteroids may interact with retinoid x-receptors (RXRs) of mammals [27] which in are dimerisation partners for a number of other nuclear (steroid) receptors [28,29].

4.1. The doses of Ecd used in the present study

Doses of Ecd taken up by the rats appear to be relatively high. A pharmacologic rule says that on the basis of per kg BW rodents require approximately 10–15-fold higher doses of compounds in order to achieve the same effects as in humans [30,31]. Augmentations of muscle mass in the humans were described under a daily intake of 200 mg/person and bodybuilders consume g-quantities of Ecd. On the basis of these considerations our lowest dose corresponded to doses suggested for normal consumers whereas the highest dose corresponded to quantities taken by bodybuilders. Hence, doses used in the present study are relevant values for the human.

It was interesting to note that Ecd at the tested doses resulted seldomly in a dose related response. This may point to a bell-shaped dose–response curve which would mean that all 3 doses were in the peak range of the bell and that theoretically even lower doses could be effective on several parameters.

In summary we showed that Ecd reduces many symptoms of the metabolic syndrome that develop in ovx rats: it decreases fat and increases muscle load. Serum HDL was higher and LDL lower in Ecd treated animals while in E2 treated ovx rats both lipoproteins were lower in ovx controls. A marked effect of Ecd was the decreasing effect in serum triglycerides which were increased by E2. Ecd was devoid of uterotrophic effects. The mechanisms of action, particu-

larly the receptors involved in mediating the effects are unknown and await further elucidation.

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