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ORIGINAL PAPER

Kerstin Fleischer · Gabi Schmidt
Theron S. Rumsey · Sonja Fritsche · Hans Steinhart
Stanislaw Kahl · Theodore H. Elsasser

Comparison of steroid hormone patterns in different fat tissues of Synovex-S implanted and control steers

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Abstract Four different adipose tissues (kidney fat, heart fat, fat over rib, tailhead fat) of six control and seven Synovex-S (containing progesterone and 17β-estradiol benzoate)-implanted steers were investigated for their profiles of progesterone, androgens, and their precursors and metabolites. The steers were implanted with Synovex-S and slaughtered after 84 days. The tissues represent different bovine depot fats. Kidney and heart fat deposit at an earlier stage of development than the other subcutaneous fat tissues was investigated. Androgens, their precursors and progesterone were analysed by GC-MS. Estrogens could not be detected by GC-MS. Resulting hormone patterns were compared between treatments and between fat depots. The statistical Kruskal-Wallis-H-test was used for comparison. The adipose tissues showed similar hormone patterns. Only progesterone showed an increased concentration in adipose tissues of implanted steers. The steroid patterns did not show the influence of exogenous steroid administration.

Keywords Steroid pattern · Synovex-S · Progesterone · Steers · Fat depots

K. Fleischer · G. Schmidt · H. Steinhart (✉)
Department of Biochemistry and Food Chemistry,
University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany
e-mail: steinhart@lc.chemie.uni-hamburg.de
Tel.: +49-40-42838–4356/7, Fax: +49-40-42838-4342

T.S. Rumsey · S. Fritsche · S. Kahl · T.H. Elsasser
USDA, ARS, ANRI, Growth Biology Laboratory Beltsville, MD 20705, USA

Current address:
T.S. Rumsey, 76 Woodland Hills Rd., Asheville, NC 28804, USA

Current address:
S. Fritsche, Unilever Research & Development Vlaardingen, The Netherlands

Introduction

Implants containing sex steroids or other anabolic steroid agents have been widely used by the United States of America cattle industry for more than 25 years. Anabolic steroid agents have growth-promoting effects such as improvement of growth rate and feed conversion efficiency. In December 1985 the European Economic Community (Directive 88/146/EEC) banned the use of natural and synthetic hormones and prohibited imports of animals and meat from animals treated with hormones. However, it is not possible to detect a treatment with natural steroids in meat because of the impossibility of distinguishing between endogenous and applied natural steroid hormones [1, 2].

Various steroids have been investigated in adipose tissues previously [3, 4, 5, 6]. Most of these studies focused on a few steroid hormones only. It seems, however, prudent to determine the pattern of steroid hormones, and their precursors and metabolites [7], because the individual biosynthesis and metabolism of animals, as well as the influence of age and environment, lead to a relevant variance in hormone concentration [8, 9]. Analysing hormone patterns in fat depots could be an appropriate and successful means of detecting a treatment, because some steroid hormones show accumulation in several fat tissues. It has already been demonstrated that the fat of bulls and heifers shows higher androstenedione levels compared to their muscle tissues [10]. Studies on the distribution of lipophilic compounds other than steroids in different fat depots have found that the concentrations of pesticide residues were higher in organ fat than in subcutaneous fat [11, 12].

It seems possible that exogenous steroid hormones change their proportions to their metabolites. The difference in these ratios can be revealed by calculation of quotients; this approach has been used to test for doping in athletes' urine [13, 14].

Another approach is to monitor the accumulation of progesterone from the Synovex-S implant in fat depots during growth and finishing. It has been reported that 30

and 60 days after administration of Synovex-S, untreated steers showed higher progesterone concentrations in fat than implanted steers [3]. On the other hand progesterone concentrations of treated steers after 61 and 90 days were slightly higher than those of control steers [15]. When considering these results, the differences in ages and breeds of the animals compared in these studies have to be taken into account. The accumulation of 17β-estradiol, the second compound of Synovex-S, and 17α-estradiol, estriol and estrone are of interest in the anabolic effects of implants. Slight, but not statistically significant, increases in 17β-estradiol and estrone levels using radioimmuno assay were noticed [3]. The estrogen concentrations found in perirenal fat for 17β-estradiol lay below the GC-MS detection limit of 0.04 µg/kg (for muscle tissue) [7]. An application of the purification and isolation methods used in this study on immunoassay methods has not been yet taken place.

Development of fat depots in steers during growth and finishing takes place in three phases. Visceral fat, which surrounds heart and kidney, deposits during the first phase, whereas subcutaneous fat is formed during the second phase. Finally intramuscular fat is formed during the third phase of fat accretion. Previous studies have focused on only one adipose tissue, primarily kidney fat or subcutaneous fat [16], so no comparison was possible.

The aim of this study was to investigate the influence of the exogenous supply of steroid hormones from the implant Synovex-S on patterns of progesterone, androgens, their precursors and metabolites. The main focus was the determination of changes in hormone biosynthesis or metabolism. By analysing the adipose tissues from the first and second phases of development it was possible to predict the deposition of steroid hormones, especially progesterone, in fat depots during fat accretion.

Material and methods

Fat samples. The animal trial was performed at the Beltsville Agricultural Research Center (USA). Thirteen young steers of the same breed (Angus×Hereford) were chosen and fed in the same way. The experimental diet was a total mixed diet consisting of 40% silage and 60% concentrate mix. The silage portion of the diet was a 50:50 mixture of corn silage and grass silage, and the concentrate was made up of corn grain, soybean meal, molasses, salt, minerals and vitamins [17]. When the steers were 7 months old and weighed approximately 283 kg, seven of the steers were implanted under the skin on the back side of the ear with Synovex-S (200 mg progesterone, 20 mg 17β-estradiol benzoate). All steers were slaughtered after 84 days, at approximate weights of 408 kg and 385 kg for implanted and control steers respectively. Adipose tissue samples (kidney fat, heart fat, fat over rib, tailhead fat) were removed at slaughter and stored at –20 °C until analysis.

Steroids Androstenedione (4-androstene-3,17-dione), androsterone (5α-androstane-3α-ol-17-one), testosterone (4-androstene-17β-ol-3-one), and progesterone (4-pregnene-3,20-dione), were purchased from Serva (Heidelberg, Germany). Dehydroepiandrosterone (DHEA) (5-androstene-3β-ol-17-one), dihydrotestosterone (5α-androstane-17β-ol-3-one), epitestosterone (4-androstene-17α-ol-3-one), hydroxyprogesterone (4-pregnene-17α-ol-3,20-dione), pregnenolone (5-pregnene-3β-ol-20-one), and methyltestosterone (17α-methyl-4-androstene-17β-ol-3-one), were obtained from Sigma (Deisenhofen, Germany).

Reagents. Chloroform, diethyl ether, 1,4-dithioerythritol, ethanol, ethyl acetate, *n*-hexane, methanol, silica gel 60, and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide of analytical grade were obtained from Merck (Darmstadt, Germany). Trimethyliodosilane was obtained from Fluka (Neu-Ulm, Germany). Solid-phase extraction (SPE) cartridges (Bond Elut C_8, Bond Elut Si and Bond Elut NH_2, 500 mg, 3 mL each), 20-mL polypropylene reservoirs and vacuum manifold were obtained from Varian (Darmstadt, Germany).

Extraction of steroids. Fat tissue (5 g) was melted using a microwave oven for 5 min. A 30-mL volume of *n*-hexane was added to the warm fat. The mixture was homogenised with a rod homogeniser (Ultra Turrax, Jahnke and Kunkel, Staufen, Germany). After cooling to room temperature the homogenate was centrifuged at 2000 *g* for 10 min. The supernatant was transferred into a mixing cylinder. A 7-mL volume of ethyl acetate was added to the residue and homogenised with a glass rod. The homogenate was centrifuged again. The supernatant was decanted and evaporated by using a rotary evaporator (45 °C). The residue was dissolved in 5 mL *n*-hexane and combined with the *n*-hexane layer.

Defatting with silica gel column. Silica gel 60 was dried for 4 h (100 °C) and thereafter 20% water was added. For an equal distribution of the water, the silica gel was rolled overnight. A volume of 20 mL *n*-hexane was added to 8 g silica gel 60 and then transferred to a chromatographic column (length 300 mm, NS 14/23, ID 10 mm). The column was conditioned with 25 mL *n*-hexane. The *n*-hexane sample extract was applied to the column. The mixing cylinder and the column were rinsed with 35 mL *n*-hexane, 20 mL *n*-hexane/ethyl acetate (90:10, v/v), and 20 mL *n*-hexane/ethyl acetate (75:25, v/v). The steroids were eluted with 25 mL methanol into a 50-mL pear shape flask. The methanol layer was evaporated to 10 mL by using a vacuum evaporator (45 °C). After addition of 60 mL methanol and 20 mL water, residual fat was removed by extraction with *n*-hexane. The clean-up of the extracts by SPE using C_8-cartridges has been described previously [7, 18].

Separation, clean-up and GC-MS analysis of steroids. Clean up of steroids, the derivatisation to trimethylsilyl ethers and GC-MS analysis have been described previously [7, 18].

Statistics. Comparisons of hormone concentrations of different fat tissues and between treatments were performed by the non-parametric H-test according to Kruskal and Wallis for two independent samples using the computer program SPSS (Base System and Professional Statistics, Version 9.0 for Windows). Significance was assumed at $P<0.05$.

Results and discussion

Considering steroid hormone concentrations of the implanted steers compared to control animals, time of slaughter is of importance. Synovex releases steroids until 80 days after implantation [19, 20]. Approximately 25% of the original implant dose remained in the ear 60 days after implantation and this residual amount was absorbed linearly at the rate of approximately 0.15% of the original dose per day [19]. The steers in the current study were slaughtered on day 84, so the implant was supposed to release steroids continuously to this point. Indeed the absorbed amount of steroids from the animals was very low by the time of slaughter.

Table 1 Steroid median concentrations of treated and control steers given in descending order (μg/kg). The range of steroid concentrations in micrograms/kilogram are given in parentheses

Steroid	Heart fat		Kidney fat		Fat over rib		Tailhead fat	
	Control *n*=6	Synovex-S *n*=7	Control *n*=6	Synovex-S *n*=7	Control *n*=6	Synovex-S *n*=7	Control *n*=6	Synovex-S *n*=7
Pregnenolone	10.05 (4.75–16.20)	8.75 (4.89–16.18)	13.78 (7.30–25.13)	10.21 (5.18–13.78)	8.89 (6.01–13.33)	10.16 (7.53–13.26)	8.47 (5.67–12.74)	6.10 (4.52–11.68)
Progesterone	0.58 (0.24–0.82)	1.34 (0.64–3.25)	1.03 (0.61–2.19)	2.41 (1.58–9.91)	0.82 (0.50–0.98)	1.91 (1.28–2.86)	0.62 (0.46–0.68)	1.75 (0.55–2.52)
Androstenedione	1.42 (0.82–2.28)	1.14 (0.21–3.45)	1.67 (0.87–3.13)	1.60 (0.54–3.05)	1.58 (1.11–2.71)	1.31 (0.15–2.32)	0.56 (0.29–1.43)	0.81 (0.13–1.83)
DHEA	1.36 (0.57–1.72)	1.07 (0.33–2.70)	1.64 (0.78–8.68)	1.08 (0.70–2.61)	1.01 (0.39–5.70)	1.54 (0.48–2.01)	1.47 (0.60–1.66)	0.61 (0.31–1.93)
cis-Androsterone	0.27 (0.20–0.44)	0.26 (<0.10–0.48)	0.38 (0.29–0.69)	0.59 (0.40–0.79)	0.34 (0.12–0.50)	0.50 (0.23–1.08)	0.37 (0.18–0.56)	0.26 (0.14–0.61)
Dihydrotestosterone	0.20 (<0.10–0.49)	0.19 (<0.10–0.24)	0.27 (0.13–4.75)	0.23 (<0.10–0.80)	0.31 (0.23–2.51)	0.38 (0.17–1.50)	0.27 (0.13–0.74)	0.23 (0.11–0.61)
Epitestosterone	0.20 (0.12–0.62)	0.61 (0.14–0.96)	0.44 (0.24–1.04)	0.40 (0.21–0.93)	0.19 (<0.10–0.55)	0.51 (0.14–0.59)	0.52 (0.14–0.71)	0.25 (<0.10–0.85)
Testosterone	<0.10	<0.10 (<0.10–0.12)	<0.10 (<0.10–1.95)	0.14 (<0.10–1.26)	0.10 (<0.10–0.34)	<0.10 (<0.10–0.18)	<0.10	<0.10 (<0.10–0.27)

DHEA Dehydroepiandrosterone, < steroid content below detection limit

Profiles of progesterone, androgens, precursors and metabolites

Profiles of steroid hormone concentrations in different fat tissues were similar (Table 1). The limited number of animals investigated, the sometimes large variations in concentrations within one type of fat and the fact that some of the determined steroid hormone concentrations were below the detection limit made it necessary to use medians instead of the arithmetic mean. To visualize the wide variations the ranges instead of sample standard deviation are given as well.

All control steers showed the same descending order of steroid concentrations. Pregnenolone was the main compound, followed by androstenedione, DHEA, and progesterone. Dihydrotestosterone, *cis*-androsterone, and epitestosterone were in the same order of magnitude, whereas testosterone showed the lowest concentrations. The treated steers showed a similar concentration pattern, with the exception of progesterone. The percentage increase of the progesterone amounts of the implanted steers compared to the control steers did not result in constant percentage decrease of the other steroid hormones investigated.

It was essential to compare the different fat types among each other within one treatment group, and the control versus implanted animals for each of the tissues individually by means of these data. For a consecutively precise comparison the precursors, effect-stages and the metabolites were regarded separately.

Comparison between treatments within the fat tissue types

Pregnenolone, dehydroepiandrosterone, and androstenedione were the main compounds in fat tissues of control steers. Dehydroepiandrosterone and androstenedione are precursors of the potent androgens testosterone and its metabolite dihydrotestosterone. Pregnenolone is also precursor for progesterone. Fat seems to store lipophilic precursors. This is consistent with observed increased amounts of androstenedione in fat of calves (males and females) and adult beef cattle (bulls and heifers) of mixed breeding [10]. There was no regular asymptotic two-sided significance between these three steroids between treatments. The application of progesterone did not result in an increase or decrease of biosynthesis of precursors.

Furthermore, there were no regular significant differences of hormones testosterone or for its metabolites dihydrotestosterone, *cis*-androsterone and epitestosterone. Significant differences for androgens resulted, however, from individual variances.

In contrast to these steroid hormones, progesterone showed a significant difference between control and implanted steers in each fat tissue, so it had a regular significance. The same conclusion regarding increased progesterone concentrations in steers has already been demonstrated [15]. The progesterone concentrations of the control steers increased from 2.48±1.61 μg/kg up to 3.40±1.32 μg/kg at 61 days after Synovex-S implantation and 3.67±2.25 μg/kg after 90 days. However, decreased progesterone concentrations during the implantation period were noted [3]. The investigated perirenal fat of control steers contained 4.55±0.79 μg/kg progesterone.

Table 2 Kruskal-Wallis-H-test between kidney fat and the other investigated adipose tissues of the implanted animals

Steroid	Fat type	Rank sum	Significance	Fat type	Rank sum	Significance	Fat type	Rank sum	Significance
Pregnenolone	Kidney fat	49	0.655	Kidney fat	60	0.338	Kidney fat	53	0.949
	Fat over rib	56		Tailhead fat	45		Heart fat	52	
Progesterone	Kidney fat	64	0.142	Kidney fat	69	0.035*	Kidney fat	40	0.110
	Fat over rib	41		Tailhead fat	36		Heart fat	65	
Androstenedione	Kidney fat	63	0.180	Kidney fat	67	0.064	Kidney fat	46	0.406
	Fat over rib	42		Tailhead fat	38		Heart fat	59	
DHEA	Kidney fat	49	0.655	Kidney fat	62	0.225	Kidney fat	48	0.565
	Fat over rib	56		Tailhead fat	43		Heart fat	57	
cis-Androsterone	Kidney fat	62	0.255	Kidney fat	72	0.013*	Kidney fat	30	0.004*
	Fat over rib	43		Tailhead fat	33		Heart fat	75	
Dihydrotestosterone	Kidney fat	41	0.142	Kidney fat	51	0.848	Kidney fat	47	0.482
	Fat over rib	64		Tailhead fat	54		Heart fat	58	
Epitestosterone	Kidney fat	52	0.949	Kidney fat	62	0.225	Kidney fat	60	0.338
	Fat over rib	53		Tailhead fat	43		Heart fat	45	
Testosterone	Kidney fat	56.5	0.609	Kidney fat	63	0.180	Kidney fat	43	0.225
	Fat over rib	48.5		Tailhead fat	42		Heart fat	62	

* Asymptotic two sided significance $P<0.05$

Thirty and sixty days after implantation with Synovex-S progesterone concentrations were 2.96±0.60 µg/kg and 2.62±0.27 µg/kg, respectively.

The progesterone concentrations in the present study were lower than in the mentioned papers. One reason could be the different races and breeding conditions of the steers. In contrast to these earlier studies the differences between the controls and the implanted animals were more noticeable in the present study. Hence, it can be concluded that increased progesterone concentration of the implanted steers was an effect of progesterone administration. Nevertheless, it would not be possible to detect a treatment without looking at control animals of the same breed and conditions, because of the great individual range of the steroid hormone contents and overlapping of progesterone contents of implanted and control steers.

Comparison between the fat tissue types

The concentration of each steroid hormone was compared between the different fat tissues. The observed significant differences were unequally distributed. Table 2 shows exemplarily the rank sums and the resultant unequally distributed significances ($P<0.05$) of the kidney fat of the implanted animals. Differences existed between and within the adipose tissues for progesterone. Therefore no evidence could be found that it was accumulated and stored in a different way during the stage of growing. Regarding the examined metabolites (dihydrotestosterone, *cis*-androsterone and epitestosterone) and precursors (pregnenolone, dehydroepiandrosterone, androstenedione), it would appear that metabolism and biosynthesis of androgens were the same and seemed not to be altered by the steroid supply.

Reconsidering progesterone, kidney fat had the highest concentration in control and implanted animals. After that fat over rib, heart fat, and tailhead fat followed. This range applied to both control and implanted steers. Kidney fat lies close to the cortex of the adrenal gland, the main tissue of steroid hormone biosynthesis for steers. This could be the reason for the high progesterone concentrations.

In conclusion, exogenous application of steroid hormones through Synovex-S as recommended by the supplier did not seem to have an effect on biosynthesis and metabolism of androgens. The progesterone applied, however, was accumulated. The hormone patterns of the fat depots were very similar. Fat tissues seemed to store precursors and showed low concentrations of potent androgens. The several steroid hormones showed the characteristic, individual wide ranges of concentrations and so it was not possible to distinguish between control and implanted steers.

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