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Pharmacokinetics and pharmacodynamics of injectable testosterone undecanoate in castrated cynomolgus monkeys (*Macaca fascicularis*) are independent of different oil vehicles

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Abstract: Testosterone undecanoate (TU) dissolved in soybean oil was developed in China to improve the pharmacokinetics of this testosterone ester in comparison with TU in castor or tea seed oil. As a pre-clinical primate model, three groups of five castrated cynomolgus macaques received either a single intramuscular injection of 10 mg/kg bodyweight TU in soybean oil, in tea seed oil, or in castor oil (equals 6.3 mg pure T/kg bodyweight for all preparations). Testosterone, estradiol, luteinizing hormone, and follicle-stimulating hormone as well as prostate volume, body weight and ejaculate weight were evaluated. After injection supraphysiological testosterone levels were induced. There were no significant differences in the pharmacokinetics of the three TU preparations for testosterone and estradiol. The gonadotropin levels showed a high individual variation. Prostate volumes increased equally in all groups after administration and declined to castrate level afterwards. The results suggest that TU in soybean oil produces similar effects as TU in the other vehicles. This study in non-human primates provides no objection to testing of this new preparation in humans.

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Introduction

Testosterone esters are used clinically as treatment for hypogonadism [24] and in trials for hormonal male contraception [15, 26]. Androgen administration to hypogonadal men should achieve and maintain physiological androgen levels over a certain period of time and supraphysiological levels should be avoided. For these purposes injectable testosterone undecanoate (TU) provides better pharmacokinetics and pharmacodynamics than testosterone enanthate [1, 8, 25, 27] and induces pronounced and reversible suppression of spermatogenesis [19, 27, 33]. Pharmacokinetics of formulations are known for injectable TU in tea seed oil and in castor oil and both preparations are in clinical use for treatment of male hypogonadism [e.g. 8] and in efficacy studies of hormonal male contraception [11, 19, 22].

Injection intervals are remarkably longer when TU in castor oil is used for treatment compared with TU in tea seed oil [1, 8, 11, 40]. As castor oil is not approved in Chinese medicine, TU was dissolved in soybean oil in order to investigate whether kinetics similar to the preparation in castor oil could be achieved. Castor oil provides better properties as vehicle for testosterone esters than tea seed oil, but is not part of the Chinese Pharmacopeia. Therefore soybean oil, which is

approved in Chinese medicine was used to develop a new formulation of TU which allows a similar concentration of the substance compared with castor oil.

Apart from previously performed studies in rats [10], soybean oil as a vehicle for testosterone application has not been investigated. Therefore, pharmacokinetics of the new TU preparation and the possible effects of the vehicle on the preparation have to be clarified to determine whether this preparation has any advantages or disadvantages over others. The pharmacokinetics of this new formulation have to be investigated in a pre-clinical study in non-human primates before the preparation is applied to men. Cynomolgus macaques were selected because they closely resemble the reproductive physiology, prostate architecture and circulating sex hormone patterns of men. In general, the dynamics of androgen production and metabolism are similar; in particular for peripheral aromatization [4, 12]. Previous studies in the cynomolgus macaque demonstrated the usefulness of this animal model in pre-clinical studies of testosterone preparations [27, 31, 32, 34]. We therefore compared the TU preparation in soybean oil with those in tea seed and castor oil in cynomolgus monkeys.

Materials and Methods

Animals

Fifteen castrated cynomolgus macaques (*Macaca fascicularis*) (4.54–9.86 kg body weight, castrated between 9 months and 12 years prior to the study was performed) were housed individually under a 12:12-hour day:night regimen in a temperature-controlled environment. All animals were fed species-specific pelleted food with a daily supplement of fresh fruits and had unlimited access to tap water. Housing and exercise conditions were identical for all animals during the experimental period. The experimental work was performed in accordance with the German Federal Law on the Care and Use of Laboratory Animals (license no. G67/2001).

Testosterone preparations

Testosterone undecanoate in soybean oil at a concentration of 250 mg TU/ml was obtained from Zhejiang Xian Ju Pharmaceutical Corp. (Zhejiang, China) and was manufactured under the Chinese Pharmacopeia. TU in tea seed oil at a concentration of 125 mg TU/ml and the experimental formulation TU in soybean oil was provided by the WHO. Ampoules with a concentration of 250 mg TU (3-oxoandrost-4-ene-17β-yl-undecanoate)/ml castor oil were provided by Jenapharm GmbH & Co. KG (Jena, Germany). Tea seed oil provides a lower solubility for TU compared with castor oil or soybean oil [1]. A higher amount than 125 mg/ml dissolved in this vehicle leads to precipitation of TU. The limited solubility of TU in tea seed oil was one of the reasons why the new formulation in soybean oil was developed. Consequently, injection volumes differ between TU in tea seed on the one hand and TU in castor oil or soybean oil on the other. However, these volume differences should not have any influence on the absorption rate and subsequently on the T effects in our study design [see 2].

Experimental design and sample collection

The 15 castrated cynomolgus macaques were randomly divided into three treatment groups as shown in Table 1. At baseline, blood was sampled, body weight was recorded and prostate volumes were obtained by transrectal ultrasonography 7 days prior to injection [36]. On study day 0 the monkeys were given a single injection of 10 mg/kg body weight of the respective TU preparation containing 6.3 mg/kg pure testosterone. Serum samples were obtained at days: 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, 19, 22, 25, 28, 31, 34, 38, and then weekly until day 159. Afterwards an additional sample was obtained at study day 189. Blood was drawn from sedated animals by venipuncture of the cubital or saphenous vein, and cooled blood (4°C) was allowed to clot overnight before it was centrifuged twice and stored at −20°C until hormone analysis. Ejaculates were collected by an

Table 1. Treatment groups

Preparation	TU in soybean oil	TU in tea seed oil	TU in castor oil
Dosage of preparation (mg/ml)	250	125	250
Group size	5	5	5
Administration: single intramuscular injection	Adjusted to 6.3 mg pure T/kg	Adjusted to 6.3 mg pure T/kg	Adjusted to 6.3 mg pure T/kg

established procedure of electric rectal stimulation [36] from animals sedated with ketamine hydrochloride (10–15 mg/kg). Ejaculates were collected beginning at study day −7 (baseline), and afterwards from study day 6 onwards in weekly intervals up to study day 159 and finally at study day 189. Ejaculate weights were measured as biological parameter of testosterone action and prostate function [27].

Hormone determinations

All hormone determinations from one animal were performed in a single run to avoid interassay variations. Testosterone was determined from ether-extracted serum using an established radioimmunoassay [27, 34]. The intra-assay coefficient of variation (CV) was 5.2% and the interassay CV was 8.7%. Estradiol levels were measured by utilizing an Autodelfia 1235 (Perkin Elmer, Freiburg, Germany). The intra- and interassay CV were 4.3 and 2.9% respectively.

Serum luteinizing hormone (LH) was measured by an established *in vitro* bioassay based on murine Leydig cells as previously described [37]. The assay was re-validated for this study by showing that the high levels of serum testosterone in TU-treated monkeys did not interfere in the bioassay. Essentially the same results were obtained when testosterone, progesterone or 17-OH progesterone were measured at the end of the incubation with the Leydig cell preparation (data not shown). The intra- and interassay CVs were below 10% respectively.

Serum follicle-stimulating hormone (FSH) was determined by a double antibody radioimmunoassay using a recombinant preparation of cynomolgus monkey FSH described previously [30] and calibrated against the cynomolgus WP-XV-104C pituitary FSH preparation [20] as the standard. The first antibody was a monoclonal anti-human FSHβ antibody obtained from Immunotech (Marseille, France) used at a 1:120,000 dilution. Bound/free separation was performed by adding a goat anti-mouse antiserum (1:100, Dako, Glostrup, Denmark) and mouse gamma globulins (1:1000, ICN, Eschwege, Germany). The sensitivity of the assay was 1.6 ng/ml. The intra- and interassay CV were below 5%.

Prostate volumes

The prostates were examined by transrectal ultrasonography using a mechanical, biplanar 7.5-MHz sector scanner (Siemens, Sonoline SL2, Erlangen, Germany) as previously published [16–18]. Total prostate volumes were measured at study day −7 (baseline), 69, 91 and 190. All scans were performed by the same investigator, who was blinded to the experimental status of the animals. In previous experiments coefficients of variation have been shown to be 6–9% within one observer [16].

Statistical analysis

Data of areas under the curve (AUC), areas under the first moment curve (AUMC) mean residence time (MRT), terminal elimination (elimination rate), half-life, maximal testosterone concentration (C_{max}), and the time to reach maximum testosterone concentration (T_{max}) were analyzed by one-way ANOVA and hormone data were analyzed applying two-way ANOVA. All data were expressed as mean ± SEM. Computations were performed using the statistical software package SIGMASTAT 2.03 (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered to be statistically significant.

Results

Bodyweight

Animals exhibited stable or slightly but not significantly decreasing body weights during the experimental period. The animals showed no change in behavior; food and water intake was also normal. No significant differences in bodyweight changes were found between the three treatment groups.

Testosterone pharmacokinetics

The individual testosterone profiles of the animals after the TU injections varied remarkably in all three treatment groups (Fig. 1). Immediately after injection of TU the mean serum levels of testosterone increased in all treatment groups (Fig. 1) and supraphysiological values were induced. The highest levels were achieved with TU in tea seed and in soybean oil, while the increase in those monkeys treated with TU in castor oil was more moderate (Fig. 1, Table 2). The testosterone levels declined continuously and reached the normal range 60–70 days post-injection. TU in soybean oil produced higher testosterone values between days 40 and 80. Afterwards testosterone levels decreased to castrate levels, which were observed around study day 100 (Fig. 1, Table 2).

The higher values achieved by administration of TU in soybean oil, in particular in the period between study days 50 and 80, are reflected by the

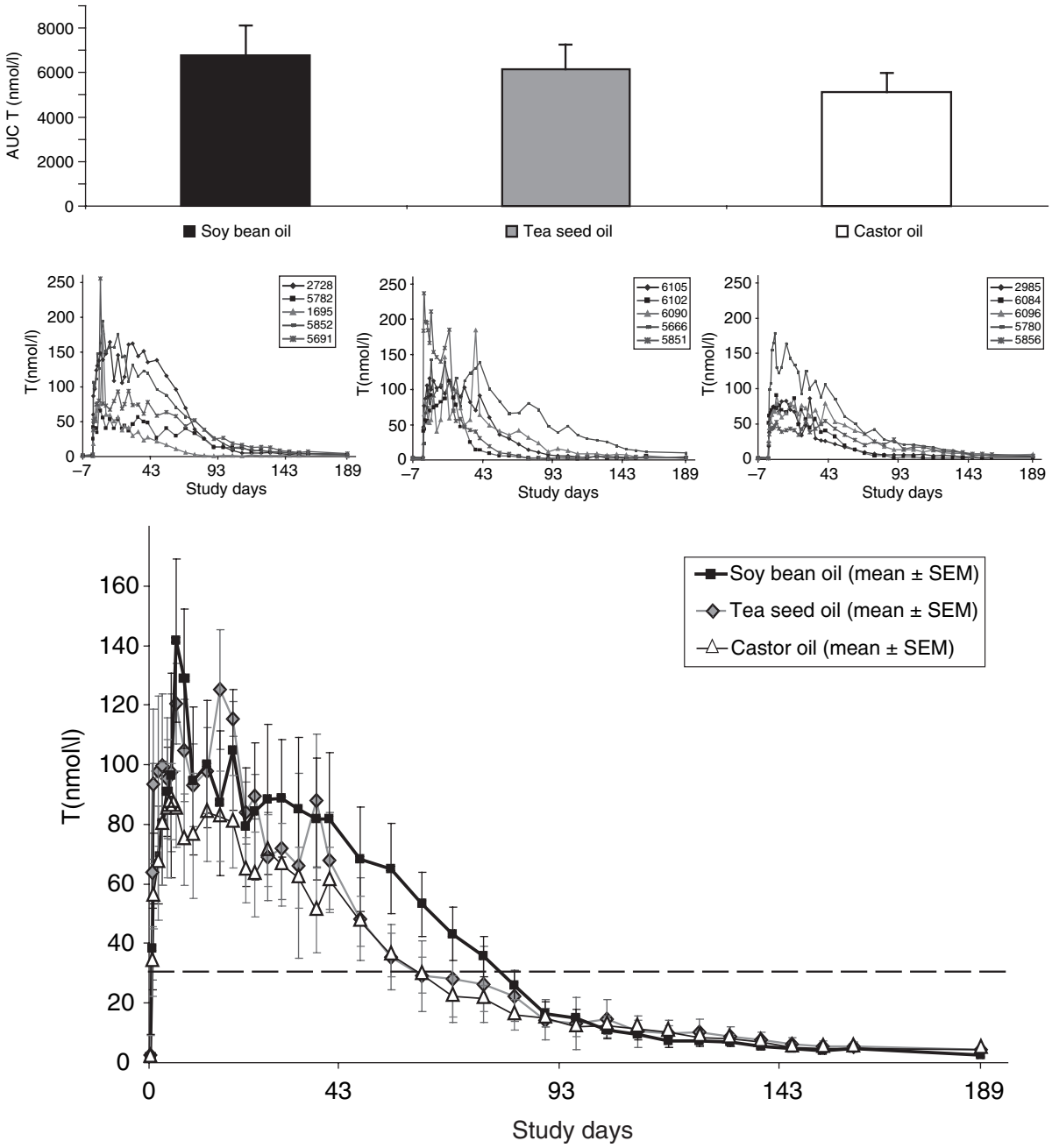

Fig. 1. Testosterone pharmacokinetics. In all groups supraphysiological values were induced by TU administration. The testosterone levels declined continuously and reached the normal range after 60–70 days post-injection. TU in soybean oil lead to higher T values between the days 40 and 80. Castrate levels were reached again around study day 100. However no significant differences at any timepoint were found. AUCs were reflecting the slight differences of achieved testosterone levels in the three treatment groups. The individual testosterone profiles of the animals varied remarkably in all three treatment groups (small panels). The dotted lines indicate the mean baseline T concentrations for intact cynomolgus monkeys.

slightly higher AUC values in the group. Testosterone AUC of the castor oil-treated group was the lowest, while the AUC of the monkeys treated with the tea seed oil preparation achieved an intermediate state (Fig. 1, Table 3). However, statistical analysis of the pharmacokinetics of the three TU preparations exhibited no significant differences at any timepoint, neither when absolute values nor when AUCs were compared. No significant differences in the pharmacokinetics of the three formulations occurred when the parameters C_{max}, T_{max}, MRT, elimination rate and half-life were compared

Table 2. Average values of the three treatment groups of the four determined hormones (testosterone, E2, bioLH, FSH)

Study day	Testosterone (nmol/l)			E2 (pmol/l)			bioLH (IU/l)			FSH (ng/ml)		
	Soybean	Tea seed	Castor oil	Soybean	Tea seed	Castor oil	Soybean	Tea seed	Castor oil	Soybean	Tea seed	Castor oil
Base	1.3 (0.1)	2.8 (0.5)	2.0 (0.4)	46.9 (3.7)	53.2 (1.9)	50.8 (2.1)	399.5 (168.0)	314.4 (67.3)	275.4 (73.0)	148.0 (62.2)	154.8 (22.6)	138.5 (29.2)
0.5	38.2 (6.9)	64.0 (27.1)	33.9 (6.4)	55.9 (4.5)	65.9 (4.2)	50.0 (1.7)	305.3 (73.2)	346.3 (83.4)	330.8 (72.6)	131.7 (51.3)	151.4 (31.3)	138.6 (30.3)
2	69.7 (10.6)	97.8 (22.6)	67.1 (9.8)	54.9 (3.1)	69.6 (6.5)	62.4 (3.9)	299.4 (78.9)	324.6 (72.5)	340.4 (98.2)	125.1 (47.5)	140.8 (25.4)	136.4 (29.1)
4	90.9 (18.8)	95.4 (21.3)	85.2 (18.4)	65.5 (11.0)	81.8 (17.9)	68.0 (5.9)	247.1 (49.8)	273.8 (66.6)	323.7 (70.8)	133.1 (49.3)	150.7 (35.2)	138.0 (25.6)
6	141.6 (30.7)	120.4 (23.6)	85.1 (11.4)	78.4 (6.0)	97.9 (18.7)	74.3 (4.2)	239.3 (30.5)	366.9 (91.3)	318.0 (53.7)	124.0 (37.2)	170.0 (40.4)	145.9 (27.8)
16	87.1 (19.1)	125.1 (12.9)	82.5 (14.8)	79.3 (10.2)	90.6 (9.0)	69.9 (7.3)	141.7 (66.0)	287.9 (82.2)	239.3 (73.4)	47.0 (25.3)	151.1 (39.2)	128.5 (37.9)
27	88.3 (20.5)	69.1 (6.7)	71.1 (12.6)	81.1 (8.5)	90.8 (8.8)	74.4 (6.7)	154.0 (68.4)	339.0 (101.2)	288.7 (91.2)	65.2 (30.0)	170.9 (25.0)	154.2 (51.5)
34	85.2 (17.6)	66.2 (15.3)	62.2 (12.4)	79.4 (7.8)	105.3 (14.9)	74.2 (7.2)	210.8 (67.1)	280.8 (57.7)	382.7 (125.0)	83.7 (33.8)	155.3 (14.2)	215.3 (51.2)
48	68.4 (19.6)	48.2 (14.6)	47.3 (9.8)	63.8 (8.0)	80.8 (10.8)	62.4 (7.7)	293.1 (96.3)	230.2 (69.3)	481.2 (110.5)	94.9 (37.0)	125.2 (17.6)	242.6 (53.8)
69	43.2 (9.6)	28.1 (10.6)	22.2 (5.4)	47.7 (5.3)	59.9 (10.4)	48.6 (5.2)	236.8 (76.9)	281.3 (50.4)	509.3 (84.3)	161.1 (30.5)	161.8 (32.9)	217.5 (36.0)
91	16.3 (4.3)	14.3 (7.8)	15.1 (4.5)	36.1 (3.5)	52.3 (6.1)	45.1 (4.5)	428.3 (76.5)	354.5 (62.9)	523.8 (48.7)	200.7 (52.3)	184.5 (31.3)	209.1 (34.5)
139	5.5 (1.1)	7.6 (2.9)	6.8 (1.4)	30.7 (5.3)	46.1 (2.3)	39.0 (3.5)	455.2 (129.3)	452.1 (82.2)	443.8 (88.9)	198.5 (58.4)	234.9 (43.2)	206.2 (33.5)

Values are mean (SEM).

(Table 3). After study day 90 the pharmacokinetic profiles of the preparations were almost identical in all animals.

Estradiol

The estradiol levels paralleled the profiles of testosterone in the animals. After an increase following the injection, estradiol levels again decreased with time (Table 2). The highest mean values were achieved in the group treated with TU in tea seed oil (~100 pmol/l), while the values in both other groups were slightly but not significantly lower (<100 pmol/l). The AUCs for estradiol did not reveal significant differences among the groups (Table 3).

Gonadotropins

An overall FSH suppression was observed only in the soybean oil-treated group while in the other groups FSH levels maintained generally a constant level. A similar result was found for the LH values. AUC data for LH and FSH were not significantly different between the three treatment groups. In contrast to the expected LH decrease, some individuals responded to high testosterone levels with constant or increasing values. These animals were identical with those whose FSH levels failed to respond. Thus the monkeys could be divided into responders and non-responders. As they are distributed randomly across the different treatment groups, no correlation was found related to the treatment or any other parameter (maximal testosterone levels, age differences, differences of bodyweight). However, although no significant differences were found between the groups when comparing absolute data and AUCs (Table 3), the levels in the three treatment groups appeared different (Table 3). Overall, the suppression of LH by testosterone administration was more effective than that of FSH. Suppression of gonadotropins seems to be strongest in the soybean oil group, intermediate in the castor oil group and weakest in the group treated with TU in tea seed oil. (Table 2, Fig. 2).

Prostate volumes

In most animals the greatest volume was observed on study day 69, in some animals on study day 91. The prostate volumes in all animals had almost dropped to baseline levels 190 days after TU administration. If the average values for the groups are compared, no significant differences concerning the effects of the different TU preparations occurred at any timepoint (Fig. 3).

Table 3. Pharmacokinetics of the three treatment groups of the four determined hormones (testosterone, E2, bioLH, FSH)

	Testosterone (nmol/l)			E2 (pmol/l)			bioLH (IU/l)			FSH (ng/ml)		
	Soybean	Tea seed	Castor oil	Soybean	Tea seed	Castor oil	Soybean	Tea seed	Castor oil	Soybean	Tea seed	Castor oil
C_{max}	141.6	125.1	87.5	81.1	105.3	74.4	455.2	452.1	523.8	200.7	234.9	242.6
Min	2.7	4.3	4.5	30.7	46.1	39.0	141.7	230.2	239.3	47.0	125.2	128.5
Median	65.1	64.0	47.3	63.8	80.8	62.4	247.1	324.6	340.4	124.6	156.2	151.9
Mean	87.8	89.4	69.0	69.8	85.3	66.9	236.3	306.2	338.1	95.3	152.5	161.0
SEM	9.8	9.0	6.5	4.9	6.0	3.8	28.5	20.4	31.8	15.5	6.3	17.8
T_{max}	8.2	13.8	17.6									
AUC	6793	6134	5119	7304	9464	7591	42,549	45,934	60,883	20,248	24,252	27,788
AUC (SEM)	3274.0	1247.5	975.8	1875.0	2632.0	1668.0	11,608	9721.0	11,178	5872.0	4836.0	6003.0
AUMC	302,971	273,820	242,396	–	–	–	–	–	–	–	–	–
MRT	44.2	40.7	46.7	–	–	–	–	–	–	–	–	–
Elimination rate	0.0193	0.0195	0.0167	–	–	–	–	–	–	–	–	–
Half-life (days)	38.3	40.8	46.3	–	–	–	–	–	–	–	–	

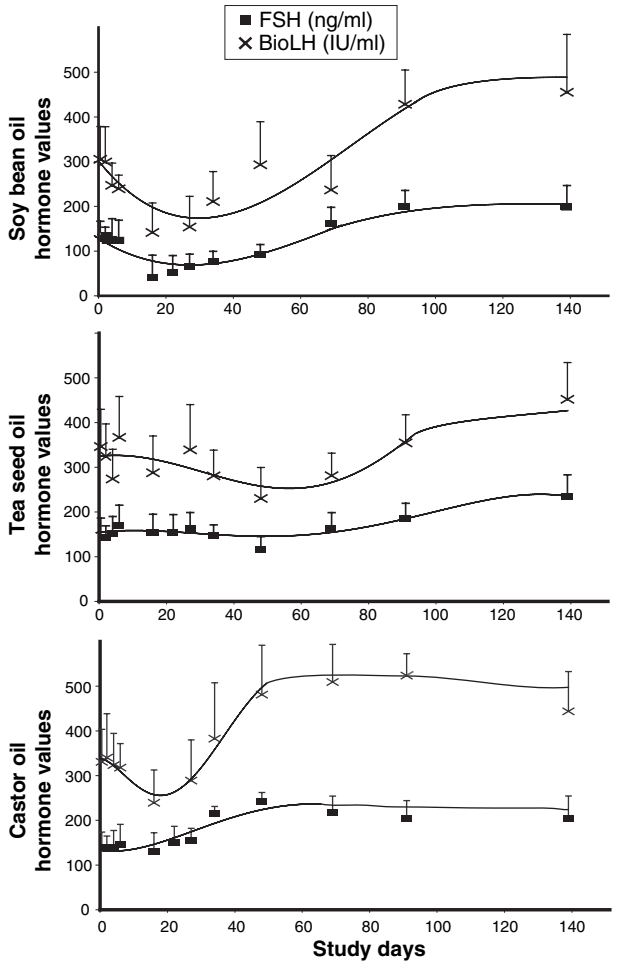

Fig. 2. Hormone levels and gonadotropin profiles. In the soybean oil-treated group the strongest FSH suppression was observed. No significant differences were found between the groups. A similar result was found for the analysis of LH values.

Ejaculates

Ejaculate weight increased continuously after TU application. In the groups treated with the tea seed or the soybean preparation a maximum weight was measured between study days 70 and 90, thereafter they declined again (Fig. 4). In the group treated with TU in castor oil the absolute amount appeared lower than in the two other groups. Two peaks occurred, the first at study day 40, the second between study days 70 and 90 (Fig. 4). The general profile was similar in all animals, but the monkeys showed remarkable individual variation. Comparison of the total ejaculate weights of the treatment groups revealed no significant differences at any time point (Fig. 4).

Discussion

Independent of the vehicle used to dissolve the TU, in all treated animals an increase in supraphysiological testosterone values was observed. In the course of the study no significant differences between the three treatment groups were found for any parameter investigated. Most likely the individual variability may influence the results. As size of all groups was identical, the mean values should be comparable with each other. By comparing the mean values to the median values we show that there is no significant trend difference between these two values for all hormones measured. Therefore we do not assume that the individual variability confounds our findings. The effect of a single TU injection in the castrated cynomolgus macaque appeared to be independent of the vehicle used. Testosterone as well as estradiol profiles, bodyweights, prostate volumes and

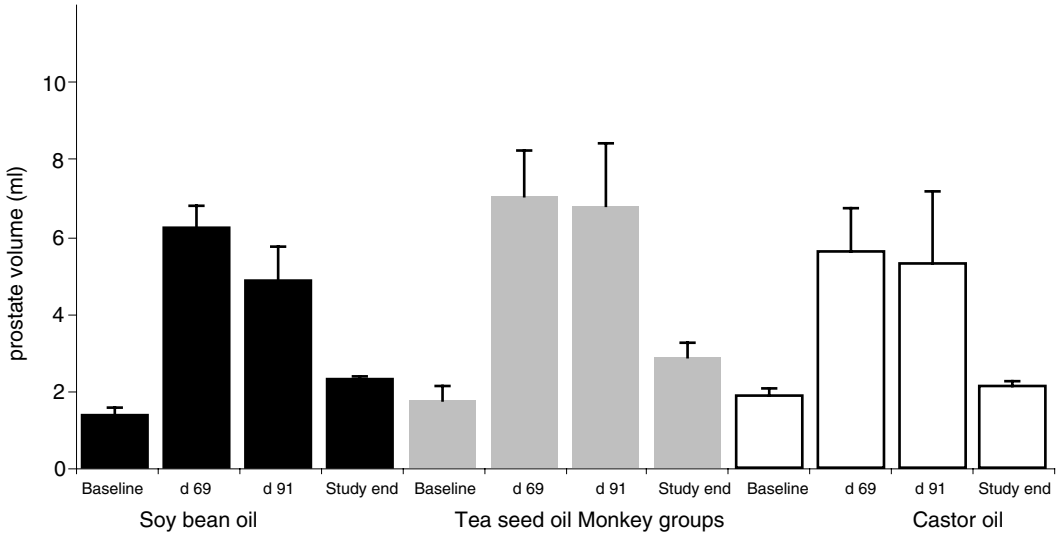

Fig. 3. Prostate volume changes. The prostate volume increased after TU administration in all treatment groups and was back to the castrate level at study day 190. No significant differences were observed between the groups.

ejaculate weights were similarly influenced in all groups.

The finding that TU administration resulted in almost identical pharmacokinetic profiles when TU in tea seed oil or TU in castor oil was applied to the macaques seems to contradict earlier results in humans from our group, demonstrating that TU in castor oil provided a longer half-life than TU in tea seed oil in hypogonadal men treated repeatedly with 1000 mg [1]. Although in the present study we also observed half-life differences, they were not proved to be statistically significant. The differences seen in humans were attributed to different properties of the vehicles, the different TU concentrations in the preparations or the different volumes administered. The comparison between the results from hypogonadal men and castrated monkeys from the current experiment is restricted. The fact that we cannot observe such differences in the current study might be because of species differences (monkeys vs. humans). The doses chosen, although similar to the doses used in humans on a weight basis, caused much higher initial serum testosterone levels in the monkeys [27]. The values are grossly supraphysiological in the monkeys, while they hardly exceed the upper normal range in men. Any potential differences of the three preparations which might have become apparent with lower doses were blurred by the supraphysiological serum levels. That species differences are of importance is corroborated by a study in rats when testosterone dissolved in soybean oil was not able to maintain LH suppression in castrated animals for longer than 14 days [10].

Prostate volume and secretion also reflect the status of androgenization and is therefore a suitable biological parameter for assessing the effects of testosterone administration. Again, no differences between the treatment groups were found, indicating that the state of androgenization achieved by TU treatment in the castrates is similar among the different preparations. As the biochemical pathways of the steroid hormones are interlinked, estradiol levels would have reflected estrogenic influences on the biochemical steroidogenic pathways and the steroid hormone levels directly and the growth of the prostate in cynomolgus macaques [12–14, 17, 21, 35]. The putative phytoestrogenic compounds of the soybean oil vehicle would have been present and affected the steroid balance in the animals [23, 29, 39]. The lack of difference in prostate volume increase between the groups also indicates that no additional phytoestrogenic effects are caused by the soybean oil during the experimental period.

As in other mammals, castration of monkeys results in rising gonadotropin levels [6, 7, 38], although with great variability [3]. The administration of testosterone should lead to a suppression of LH and FSH although inhibin is not present in castrates. However, such suppression was not observed in all monkeys. Some of the animals did not respond to the testosterone treatment at all. Also in a previously published study it was observed that gonadotropin suppression after treatment of castrated macaques with TU was much more variable than after treatment with testosterone enanthate [27]. This

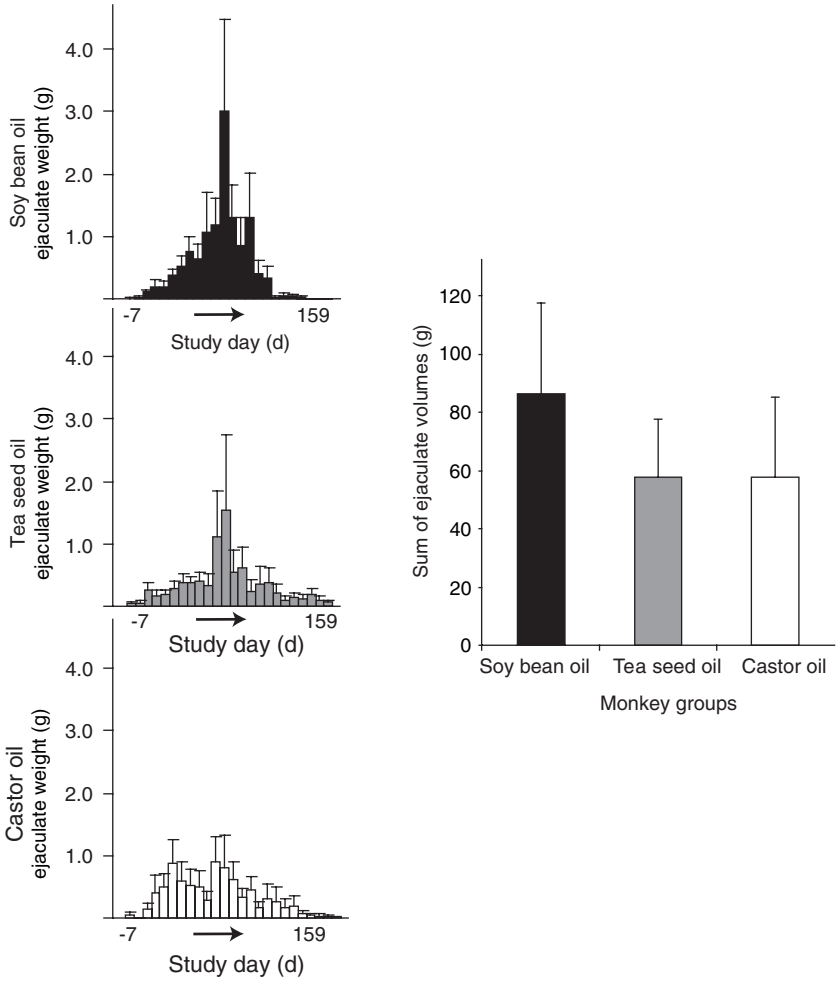

Fig. 4. Ejaculate weight increased after TU application. Ejaculates were collected beginning at study day −7 (baseline), and afterwards from study day 6 onwards in weekly intervals up to study day 159 and finally at study day 189. Each column represents the mean ejaculate weight in the three treatment groups per collection day. In the groups that were treated with TU in tea seed or soybean oil a maximum weight was measured between study days 70 and 90 before the ejaculate amounts began to decline again. In the group treated with TU in castor oil the absolute amount appeared lower than in the two other groups and two peaks occurred, the first at study day 40, the second between study days 70 and 90. The comparison of the AUCs of the treatment groups did not reveal significant differences.

phenomenon of testosterone resistance is also known to exist in Klinefelter patients, in whom, despite normalization of circulating testosterone levels, gonadotropin levels often remain elevated [5, 9, 28]. This kind of androgen resistance was not reported before in long-term castrated monkeys. Dubey et al. [7] demonstrated that in rhesus monkeys steroids were not able to suppress gonadotropins immediately after castration and suggested that inhibin is mainly responsible for the response in the period immediately following castration. In conclusion, our data show that the pharmacokinetics of injectable TU formulations are independent of the vehicle used in cynomolgus macaques.

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