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# Long-term feeding of hydroalcoholic extract powder of *Lepidium meyenii* (maca) enhances the steroidogenic ability of Leydig cells to alleviate its decline with ageing in male rats

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**Summary**

This study examined whether feeding hydroalcoholic extract of *Lepidium meyenii* (maca) to 8-week-old (sexually maturing) or 18-week-old (mature) male rats for more than a half year affects serum testosterone concentration and testosterone production by Leydig cells cultured with hCG, 22R-hydroxycholesterol or pregnenolone. Testosterone concentration was determined in the serum samples obtained before and 6, 12, 18 and 24 weeks after the feeding, and it was significantly increased only at the 6 weeks in the group fed with the maca extract to maturing rats when it was compared with controls. Testosterone production by Leydig cells significantly increased when cultured with hCG by feeding the maca extract to maturing rats for 27 weeks (35 weeks of age) and when cultured with 22R-hydroxycholesterol by feeding it to mature rats for 30 weeks (48 weeks of age). Overall testosterone production by cultured Leydig cells decreased to about a half from 35 to 48 weeks of age. These results suggest that feeding the maca extract for a long time to male rats may enhance the steroidogenic ability of Leydig cells to alleviate its decline with ageing, whereas it may cause only a transient increase in blood testosterone concentration in sexually maturing male rats.

**KEYWORDS**

ageing, Leydig cells, maca, rat, testosterone

## 1 | INTRODUCTION

Although *Lepidium meyenii* (maca), a plant of the brassicae family, has been traditionally used in Peruvian Andes to enhance male and female reproductive performance in human and livestock (Flores, Walker, Guimaraes, Bsid, & Vivanco, 2003; Tharakan & Manyam, 2005), it is not clear how maca works in reproductive systems.

Although it is reported that treatment with maca improves male reproductive function (Cicero, Bandieri, & Arletti, 2001; Cicero et al., 2002; Gonzales, Ruiz, Gonzales, Villegas, & Córdova, 2001a; Gonzales et al., 2001b, 2004, 2005; Zenico, Cicero, Valmorri, Mercuriali, & Bercovich, 2009; Zheng et al., 2000), its effect on blood concentrations of testosterone, one of representative hormones regulating male reproductive organs, is controversial. Treatments with the aqueous extracts of maca or gelatinised maca had no effect on blood testosterone levels in rats and men (Gonzales et al., 2002, 2003a, 2005; Rubio et al., 2006), while Oshima, Gu, and Tsukada (2003) showed that providing prepubertal male mice with maca powder for 30 days increased plasma testosterone concentration. Although administration of the alcoholic extract of maca for less than 3 weeks did not show stimulatory effects on blood testosterone levels (Gonzales, Rubio, Chung, Gasco, & Villegas, 2003b), we have reported that feeding the hydroalcoholic extract powder of maca, MACAXS™ for 6 weeks to 8-week-old male rats, increases serum testosterone concentration and enhances the

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steroidogenic ability of cultured Leydig cells (Ohta et al., 2016). As 8-week-old male rats are sexually maturing and their Leydig cells are still on the stage of development (Ariyaratne & Mendis-Handagama, 2000), it remains to be studied whether administration of MACAXS™ increases blood testosterone concentration in the sexually mature animals. Furthermore, the effects of the feeding for a longer time on changes in blood testosterone concentration and steroidogenic ability of Leydig cells by ageing also left to be investigated.

This study examined the effects of feeding MACAXS™ for about a half year on the serum testosterone level and the ability of testosterone production by cultured Leydig cells in sexually maturing and mature male rats.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

The experiments, approved by the Animal Experiment Committee of Osaka Prefecture University (Japan), were conducted with humane care, according to the Guidelines for Animal Experimentation of Osaka Prefecture University. Seven- or 11-week-old male rats of the Wistar strain, purchased from Japan SLC Inc. (Hamamatsu, Japan), were maintained at the Education and Research Center for Experimental Animal Science (Osaka Prefecture University) under specific pathogen-free environment. Two animals were housed in a cage with a 12 hr:12 hr light–dark schedule (light: 0800–2000 hr) at a temperature of 20–24°C and a relative humidity of 45%–65% with tap water and pelletised diets available *ad libitum*. For acclimatisation, the rats were provided with a widely used standard diet (Charles River Formula 1, Oriental Yeast, Tokyo, Japan) during more than 1 week, and the diets prepared for the present experiments were given thereafter. Body weights and dietary intake per cage were determined every week during the experiments.

### 2.2 | Diets containing hydroalcoholic extract of maca

The hydroalcoholic extract powder, MACAXS™ (TOWA CORPORATION K. K., Tokyo, Japan), was mixed in the diets as has been previously reported (Ohta et al., 2016). In brief, the powder was produced by extraction from dried bulbs of maca with hydrous ethanol and evaporation by spray drying. MACAXS™ was added to the diet based on AIN-93G in the rate of 2%.

### 2.3 | Experimental design

#### 2.3.1 | Experiment 1

Eight-week-old rats were randomly divided into the maca-fed or control group. The rats were given the diets with ($n$ = 8) or without the maca extract ($n$ = 8) for 27 weeks. Before providing the experimental diets and 6, 12, 18 and 24 weeks later, the rats were anesthetised with i.p. injection of 30 mg/kg body weight (bw) sodium pentobarbital (Somnopentil, Kyoritsuseiyaku Corporation, Tokyo, Japan), and 0.2-ml blood samples were collected from the jugular vein at 1100–1200 hr. The blood was immediately chilled by ice, and serum was obtained by centrifuging at 1710 g and stored at −80°C until hormone assay. After feeding the experimental diets for 27 weeks (35 weeks of age), the rats were weighed and anesthetised at 1000–1400 hr. The testes of two or three rats in each group (the maca-fed and control groups) were removed and weighed, and Leydig cells were separated and cultured as described below. The culture was repeated three or four times. Testosterone production by the Leydig cell was evaluated.

#### 2.3.2 | Experiment 2

When the rats became 18 weeks old, the rats were randomly divided into the maca-fed or control group. The rats were given the diets with ($n$ = 13) or without the maca extract ($n$ = 12) for 30 weeks. Before providing the experimental diets and 6, 12, 18 and 24 weeks later, the rats were anesthetised, and 0.2-ml blood samples were collected. The obtained sera were stored until hormone assay. After feeding the experimental diets for 30 weeks (48 weeks of age), the rats were weighed and anesthetised at 1000–1400 hr, and Leydig cells were separated and cultured as described below. The culture was repeated four or five times.

### 2.4 | Isolation and culture of Leydig cells

Leydig cells were isolated and purified by Percoll density gradient centrifugation as has been previously reported (Ohta et al., 2016). Viability of the cells and purity of Leydig cells were examined by trypan blue exclusion test and histochemical staining for 3β-hydroxysteroid dehydrogenase (Klinefelter, Hall, & Ewing, 1987). Viability and purity of the obtained cells were >95%, and >73% respectively. Isolated cells, seeded on each well (100,000 cells 200 $\mu l^{-1}$), were cultured with human chorionic gonadotropin (hCG; Puberogen 5,000 IU, Yell pharmaceutical Co., Ltd., Tokyo, Japan) or steroidogenic substrates in the 96-well plate at 37°C in a humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 2 hr. Ten IU/ml of hCG, 20 μmol/L 22*R*-hydroxycholesterol (Sigma, St. Louis, MO, USA) or 2.5 μmol/L pregnenolone (MP Biomedicals, Santa Ana, CA, USA) was included in the culture medium. Control wells of steroidogenic substrates were cultured in the same concentrations of the solvents, 0.1% ethanol (22*R*-hydroxycholesterol) and 0.025% ethanol (pregnenolone), and hCG was dissolved in the culture medium. Duplicate wells were prepared for each treatment. At the end of incubation, the medium was stored at −80°C for the determination of testosterone concentration. Testosterone productions (pg $10^4$ $cells^{-1}$ 2 $hr^{-1}$) were calculated from the average values in the duplicate wells.

### 2.5 | Hormone assay

Testosterone concentration in serum samples or culture media was measured by enzyme immunoassay (EIA) using the HRP-labelled testosterone and antitestosterone antibody according to the method described previously (Kawate et al., 2011; Ohta et al., 2016). The samples were assayed directly (culture media) or after extraction with diethyl ether (serum samples). The intra-assay and inter-assay CVs

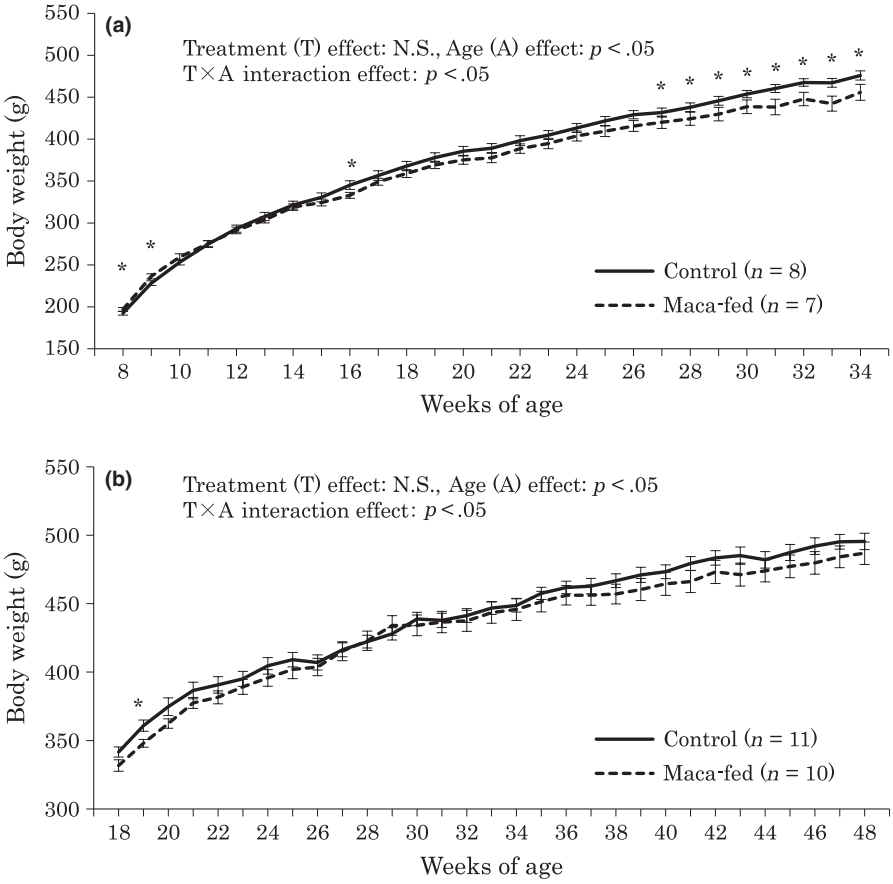


**FIGURE 1** Changes in the body weight of male rats in the maca-fed and control groups during Experiment 1 (a) and Experiment 2 (b). Values are mean ± SEM. *$p$ < .05 between the control and maca-fed groups at the same weeks of age

were <15.7 % ($n$ = 4) and <14.3% ($n$ = 10) in the serum, and <19.3% ($n$ = 4) and <15.2% ($n$ = 4 or 5; at 35 or 48 weeks of age) in the medium respectively. The minimum detection limit was 0.055 ng/ml.

### 2.6 | Statistical analysis

Values are expressed as the mean ± SEM. For body weights, dietary intake and serum testosterone concentration, the effects of treatment (controls versus maca-fed), of weeks of age and of interaction between treatment and weeks of age were all evaluated by repeated measure ANOVA. Significant difference between the respective values was analysed by the least significant difference (LSD) post hoc test (SPSS version 22 software, IBM, Somers, NY, USA). For comparison of the significant difference in testosterone production by Leydig cells and testicular weights between groups, Student's $t$-test or Welch's $t$-test was used after analysing distribution by $F$ test (Statcel software, add-ins for Excel, 3rd ed.; OMS Ltd., Tokorozawa, Japan). Values of $p$ < .05 were considered statistically significant. $p$ values between .05 and .15 were treated as indication of the tendency for differences in the data of testosterone production by Leydig cells.

## 3 | RESULT

One rat of the maca-fed group in Experiment 1, and two rats of the maca-fed and two rats of control groups in Experiment 2 died, and a mass of hair was found in the stomach at autopsy in each rat. All data of these animals were eliminated.

Figure 1 shows changes of body weight in the maca-fed and control groups in Experiment 1 (Figure 1a) and Experiment 2 (Figure 1b). In both experiments, effect of weeks of age was significant, whereas that of treatment was not. The interaction between effects of weeks of age and of treatment was significant. When compared between the control and maca-fed groups at the same weeks of age, the decreased body weight in the maca-fed group than that in the control group was found during the last 7 weeks in Experiment 1 (Figure 1a). In Experiment 2, however, the difference in body weight between the groups was not seen except at 2 weeks after the start of experiment (Figure 1b).

Figure 2 shows changes of the dietary intake in the maca-fed and control groups in Experiment 1 (Figure 2a) and Experiment 2 (Figure 1b). In both experiments, effect of weeks of age was significant, whereas that of treatment was not. The interaction between effects of weeks of age and of treatment was significant. When the values were compared between the control and maca-fed groups at the same weeks of age in Experiment 1, the dietary intake in the maca-fed group was greater than that in the control group at 9–10, 13–14, 17–18 and 22–23 weeks of age, and on the contrary, increased dietary intake in the control group than in the maca-fed group was found at 30–31, 32–33 and 33–34 weeks of age (Figure 2a). In Experiment 2, the dietary intake in the control group was greater than that in the maca-fed group at 19–20, 35–36, 43–44, 46–47 and 47–48 weeks of

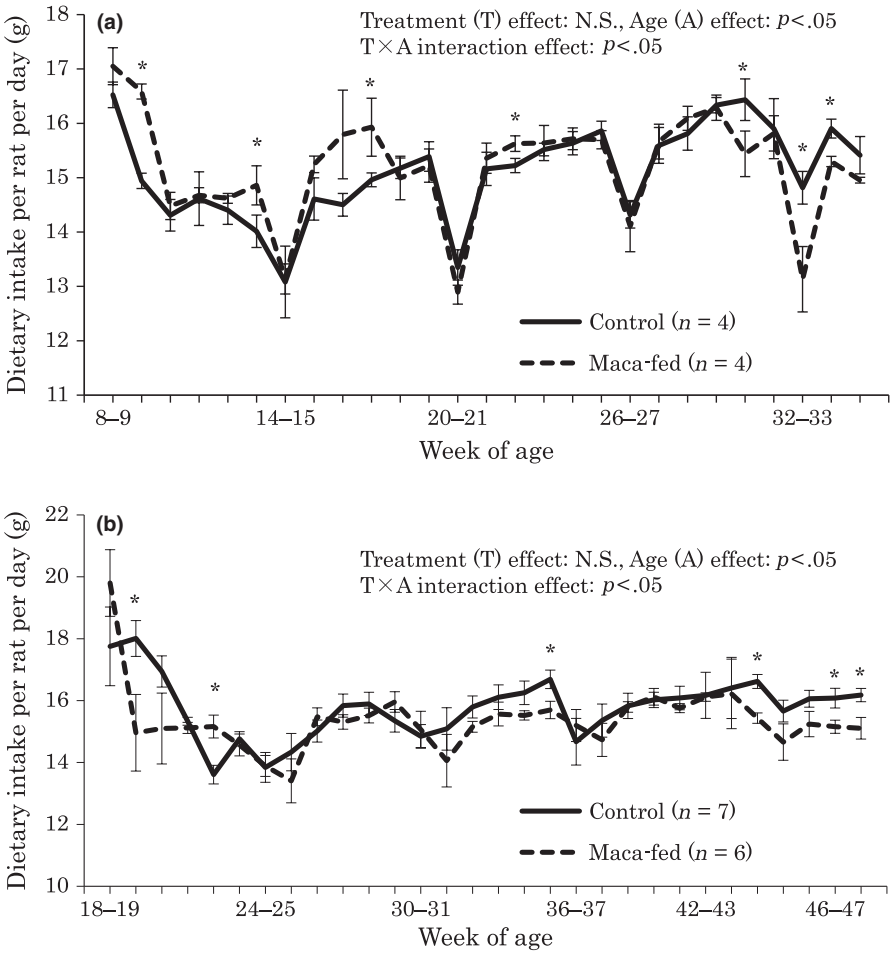


**FIGURE 2** Effect of feeding maca extract on the dietary intake in male rats during Experiment 1 (a) and Experiment 2 (b). Values are mean ± SEM. *$p$ < .05 between the control and maca-fed groups at the same weeks of age

age, and the increased value in the maca-fed group than in the control group was found only at 22–23 weeks of age (Figure 2b). When the values in each group were compared between the different weeks of age in Experiment 1, the dietary intake at 8–9 weeks of age (the week of the start of experiment) in the control group was significantly higher than that at 9–10 and 10–11 weeks of age, and the value at 8–9 weeks of age in the maca-fed group was also significantly higher than that at 10–11 weeks of age ($p$ < .05). The dietary intake before blood collection was significantly greater than that at the week of blood collection in the control (13–14 versus 14–15, 19–20 versus 20–21, 25–26 versus 26–27 weeks of age) and/or maca-fed group (13–14 versus 14–15, 19–20 versus 20–21, 25–26 versus 26–27 and 31–32 versus 32–33 weeks of age) ($p$ < .05). In Experiment 2, when the values in each group were compared between the different weeks of age, the value at 18–19 weeks of age (the week of the start of experiment) in the control group was significantly higher than that at 21–22 weeks of age, and the dietary intake at 18–19 weeks of age in the maca-fed group was significantly higher than that at 19–20, 20–21 and 21–22 weeks of age ($p$ < .05). The dietary intake before blood collection was not significantly greater than that at the week of blood collection except for two time points in the control group (23–24 versus 24–25, and 35–36 versus 36–37 weeks of age; $p$ < .05).

Figure 3 shows the serum testosterone concentration before and every 6 weeks after feeding the experimental diets to 8-week-old (Experiment 1, Figure 3a) or 18-week-old rats (Experiment 2, Figure 3b) in the maca-fed and control groups. In Experiment 1, effect of weeks of age was significant, whereas that of treatment was not. The interaction between effects of weeks of age and of treatment was significant. When the values were compared between the control and maca-fed groups at the same weeks of age, serum testosterone concentration at 14 weeks of age in the maca-fed group was significantly higher than that in the control group ($p$ < .05). In Experiment 2, on the other hand, effect of weeks of age, the effect of treatment and their interaction were not significant.

Tables 1 and 2 show testosterone production by Leydig cells cultured for 2 hr with various steroidogenic stimulants in the maca-fed and control groups in experiments 1 and 2 respectively. Mean (±SEM) testicular weights for Experiment 1 were 3.24 ± 0.06 g ($n$ = 8) in the control group and 3.17 ± 0.05 g ($n$ = 7) in the maca-fed group, and significant difference was not found in the values between groups. Testosterone production by Leydig cells tended to increase when cultured with 22R-hydroxycholesterol ($p$ = .07) or pregnenolone ($p$ = .09) and significantly increased when cultured with hCG ($p$ < .05) by feeding the maca extract (Table 1). In the rats fed the diets to 18-week-old rats for 30 weeks (Experiment 2), mean (±SEM) testicular weights were 3.14 ± 0.06 g ($n$ = 11) in the control group and 3.13 ± 0.04 g ($n$ = 10) in the maca-fed group, and significant difference was not found in the values between groups. Testosterone production by Leydig cells

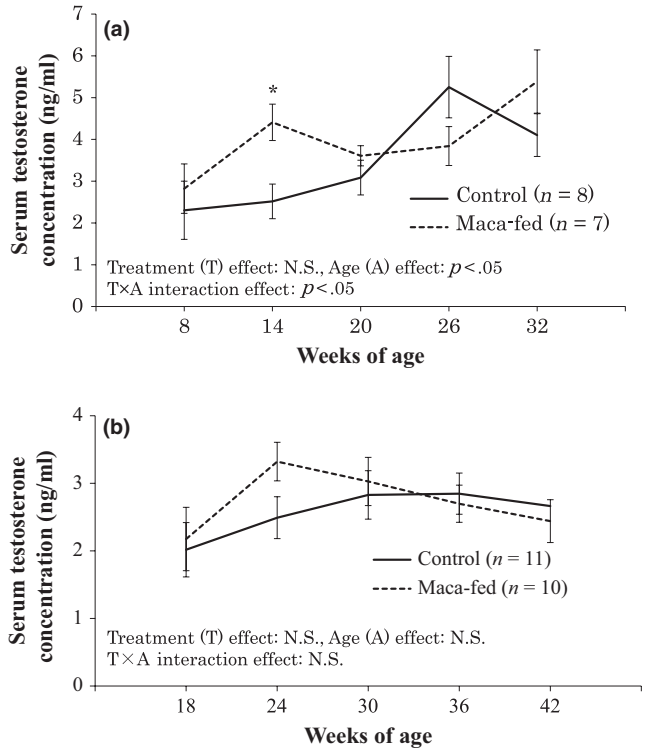


**FIGURE 3** Effect of feeding maca extract on the serum testosterone concentration in male rats during Experiment 1 (a) and Experiment 2 (b). Values are mean ± SEM $^{*}p < .05$ between the control and maca-fed groups at the same weeks of age

**TABLE 1** Effects of feeding the maca extract for 27 weeks to male rats on testosterone production by cultured Leydig cells (Experiment 1). The rats were euthanised at 35 weeks of age

| | Control | Maca-fed |
|---|---|---|
| Number of samples | 4 | 3 |
| Testosterone production (pg/$10^4$ cells/2 hr) after addition of | | |
| Non (control for hCG) | 29 ± 6 | 31 ± 5 |
| hCG | 239 ± 11 | 359 ± 18* |
| Solvent of 22R-hydroxycholesterol (0.1% ethanol) | 35 ± 13 | 28 ± 7 |
| 22R-hydroxycholesterol | 965 ± 72 | 1,165 ± 50# |
| Solvent of pregnenolone (0.025% ethanol) | 25 ± 3 | 26 ± 3 |
| Pregnenolone | 1,157 ± 47 | 1,438 ± 109# |

Diets containing the maca extract were fed from 8 to 35 weeks of age. Values are mean ± SEM.
*$p < .05$ versus controls.
#$p < .10$ versus controls.

tended to increase when cultured with pregnenolone ($p = .11$) and significantly increased when cultured with 22R-hydroxycholesterol ($p < .05$) by feeding the maca extract (Table 2). There was no difference in the testosterone production between the maca-fed and control groups when cultured with any solvents for steroidogenic stimulants in both experiments. On the other hand, when compared the testosterone production in the control group between Experiment 1 and Experiment 2, the values in Experiment 1 (35 weeks of age) were more than twofold of those in Experiment 2 (48 weeks of age) when cultured with hCG, 22R-hydroxycholesterol or pregnenolone, indicating that steroidogenic ability by Leydig cells markedly decreased with ageing from 35 to 48 weeks of age. Feeding the maca extract to 18-week-old rats for 30 weeks (48 weeks of age) alleviated the decline of testosterone production with ageing, but not to the level of the control group at 35 weeks of age.

## 4 | DISCUSSION

Feeding hydroalcoholic extract powder of maca, MACAXS™, to maturing male rats for 42 days increased blood testosterone concentration and enhances the steroidogenic ability of Leydig cells (Ohta et al., 2016). As the effects of long-term feeding of maca to animals of different ages on blood testosterone concentration and steroidogenic ability of Leydig cells are unknown, this study examined the serum testosterone concentration and testosterone production by cultured Leydig cells in both maturing and mature male rats fed with MACAXS™ for about a half year.

During the present study, three rats of the maca-fed group and two rats of the control group died with a mass of hair in the stomach. It is reported that semi-purified diets contribute to the formation of gastric hair balls in Wistar-Kyoto rats (Krugner-Higby, Wolden-Hanson, Gendron, & Atkinson, 1996). It may be possible that the diets and breed used in the present study influenced trichobezoar formation.

Body weight increased with age in both maca-fed and control groups and was decreased by the treatment with MACAXS™ during the last 7 weeks (28–34 weeks of age) in Experiment 1 (Figure 1a). During this term, the dietary intake in the maca-fed group showed a decrease or no change when compared to that in the control group (Figure 2a). These results may suggest that decreased dietary intake by the treatment with MACAXS™ caused the body weight loss during the last stage of Experiment 1. At the last stage of Experiment 2, the dietary intake in the maca-fed group also decreased or unchanged when compared with that in the control group (Figure 1b), while there was no significant difference in the body weight between the groups (Figure 2b). It remains to be studied whether, and if so, why feeding with MACAXS™ for long time nonaccidentally decreases the dietary intake in rats. Although the dietary intake decreased rapidly for several weeks after the start of feeding the experimental diets in both groups, the values were kept relatively constant during the feeding. It may be possible that the change in the diets from the standard diet during acclimatisation to the experimental diets increased the appetite by curiosity at the first few weeks. In Experiment 1, the dietary intake in both the maca-fed and control groups decreased at the week when blood samples were collected. This decrease may be due to the effects of anaesthesia and reduced blood volume. However, the decrease in dietary intake was not found in Experiment 2. Although the reason is not clear, ageing of the rats with increased weights may affect this phenomenon.

**TABLE 2** Effects of feeding the maca extract for 30 weeks to male rats on testosterone production by cultured Leydig cells (Experiment 2). The rats were euthanised at 48 weeks of age

| | Control | Maca-fed |
|---|---|---|
| Number of samples | 5 | 4 |
| Testosterone production (pg/$10^4$ cells/2 hr) after addition of | | |
| Non (control for hCG) | 15 ± 6 | 17 ± 3 |
| hCG | 107 ± 35 | 172 ± 15 |
| Solvent of 22R-hydroxycholesterol (0.1% ethanol) | 10 ± 3 | 15 ± 2 |
| 22R-hydroxycholesterol | 422 ± 116 | 747 ± 42* |
| Solvent of pregnenolone (0.025% ethanol) | 9 ± 2 | 15 ± 2 |
| Pregnenolone | 491 ± 137 | 801 ± 76# |

Diets containing the maca extract were fed from 18 to 48 weeks of age. Values are mean ± SEM.
*$p$ < .05 versus controls.
#$p$ = .11 versus controls.

Consistent with the previous report (Ohta et al., 2016), the serum testosterone concentration increased 6 weeks after feeding MACAXS™ to 8-week-old male rats when compared with controls. However, there was no difference in the value between the maca-fed and control groups at 12, 18 and 24 weeks after the feeding in Experiment 1, suggesting that feeding MACAXS™ may cause a transient increase in serum testosterone concentration in maturing male rats. On the other hand, in Experiment 2 using 18-week-old male rats, there were no effects of treatment, age and their interaction in serum testosterone concentration, indicating that oral administration of MACAXS™ to mature male rats has no effect on serum testosterone concentration. However, a possibility that feeding MACAXS™ to mature male rats may induce a transient increase in blood testosterone concentration earlier or a little later than 6 weeks remains to be clarified.

Determination of steroidogenesis in the cultured Leydig cell has been proved to be a significant measure to evaluate their steroidogenic ability. This study used hCG, which exerts luteinizing hormone (LH) action, 22*R*-hydroxycholesterol, which passes a mitochondrial membrane, and pregnenolone. Overall testosterone production by Leydig cells cultured with steroidogenic stimulants in Experiment 1 (35 weeks of age) was much greater than that in Experiment 2 (48 weeks of age), indicating that the steroidogenic ability of Leydig cells declines from 35 to 48 weeks of age. The results were consistent with the previous report that showed age-related decrease in testosterone production by Leydig cells in vitro (Chen, Hardy, & Zirkin, 2002). On the other hand, feeding MACAXS™ to either 8- or 18-week-old rats for 27–30 weeks significantly increased or tended to increase testosterone production by Leydig cells cultured with several steroidogenic stimulants. These results suggest that feeding MACAXS™ may alleviate the decline in the steroidogenic ability of Leydig cells with ageing. However, feeding MACAXS™ to 8-week-old rats significantly increased testosterone production by Leydig cells cultured with hCG, whereas feeding it to 18-week-old rats significantly increased the testosterone production cultured with 22R-hydroxycholesterol. The steps where MACAXS™ affects steroidogenesis of Leydig cells may change with the age of animals when Leydig cells were collected or feeding the maca extract was started.

The reason why enhancement in the steroidogenic ability of Leydig cells by feeding the maca extract did not reflect blood level of testosterone is not clear. However, the blood testosterone concentration is controlled by negative feedback of testicular hormones to the hypothalamus and pituitary, and this mechanism might maintain the constant blood testosterone levels. On the other hand, the prepubertal increase in gonadotrophin secretion necessary for the induction of male puberty may mainly be caused by diminution of this negative feedback action of testicular hormones (Docke, Rohde, Freitag, & Dorner, 1988). In the prepubertal or maturing stage, this diminution of androgen action might be affected by the maca treatment, and the enhanced ability of Leydig cells to produce testosterone by feeding MACAXS™ might have been reflected by increased serum testosterone concentration without enough suppression of blood LH levels. The previous report (Ohta et al., 2016) showed that the maca extract increased serum testosterone levels without changes in the serum LH concentration after 6-week treatment to maturing male rats.

Male rats show a biphasic rhythm of plasma testosterone concentration (Waite, Kershaw, Spiga, & Lightman, 2009), and the correlation of individual LH pulse with the peaks of testosterone response could not be specified because of the episodic secretion of LH and testosterone in male rats (Hakola, Pierroz, Aebi, Vuagnat, & Aubert, 1998). However, testosterone production by Leydig cells is stimulated by LH, and in the present study, serum LH levels were not determined. Further studies will be needed to clear the effect of maca on pulsatile secretions of LH and testosterone.

Although the special components of maca are the origins of flavour glucosinolates, the polyunsaturated fatty acids macaen and macamide, and lots of alkaloids as a physiologically active substance (Campos, Chirinos, Barreto, Noratto, & Pedreschi, 2013; Zha et al., 2014), the constituents which affect testosterone production are not clear.

In conclusion, this study shows that feeding MACAXS™ increases testosterone production by Leydig cells cultured with steroidogenic stimulants to alleviate its decline with ageing, but causes only a transient increase of serum testosterone concentration in maturing male rats.

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