Running head

α-Tocotrienol enhances steroidogenesis

Title

α-Tocotrienol in rice bran enhances steroidogenesis in mouse Leydig cell via increased gene expression of steroidogenic acute regulatory protein and induction of its mitochondrial translocation

Naofumi Terada,[1] Tomoaki Nagase,[1] Hiromi Kayoka,[1] Yusuke Adachi[2] and Eisuke Kato[3]*

1 Frontiers in Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

2 Department of Bioscience and Chemistry, School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

3 Division of Fundamental AgriScience and Research, Research Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

Corresponding author

*Eisuke Kato

Kita-9, Nishi-9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

E-mail: eikato@agr.hokudai.ac.jp

Abstract

Rice is a staple food in the Asian region and one of the world's major energy sources. Testosterone is a steroid hormone that maintains physical, sexual, and cognitive ability and its decline causes health problems like late-onset hypogonadism. Evaluation of various grain extracts showed rice bran to stimulate testosterone secretion from Leydig model cell. α-Tocotrienol was found as a bioactive compound in rice bran, and mechanistic analysis showed the stimulation of steroid hormone synthesis through enhanced gene expression of steroidogenic acute regulatory protein as well as inducing mitochondrial localization of the protein. Preliminary study showed increasing trend in serum testosterone levels in mice by oral intake of α-tocotrienol. These results suggest that α-tocotrienol intake may be effective in preventing symptoms caused by low testosterone levels.

Keywords

Rice bran, tocotrienol, Leydig cell, testosterone, late-onset hypogonadism

38

39 **Introduction**

40 Androgens are steroid hormone essential for male reproductive function and provide the
41 body with anabolic effects. In men, stress and aging cause a decrease in serum testosterone levels,
42 resulting in symptoms in the body, medically termed late-onset hypogonadism (LOH) (Rodrigues *et*
43 *al.* 2021). The main symptoms of LOH include sexual dysfunction, muscle weakness, obesity,
44 osteoporosis, insomnia, fatigue, poor concentration, and depression (Huhtaniemi *et al.* 2011).
45 Androgen levels are also associated with the risk of cardiovascular disease (Kloner *et al.* 2016), type
46 2 diabetes mellitus (Gianatti *et al.* 2020), and cognitive functions (Matousek *et al.* 2010), indicating
47 the importance of levels.

48 Medically, a decrease in testosterone, the primary androgen, is compensated for by
49 exogenous testosterone, called testosterone therapy. Testosterone therapy improves symptoms caused
50 by low testosterone level but is associated with adverse effects such as increased red blood cell
51 counts, male infertility, testicular atrophy and gynecomastia (Habito *et al.* 2000). Other therapies
52 include human chorionic gonadotropin and aromatase inhibitors, which indirectly increase
53 testosterone.

54 As an alternative to the medication, foods can affect androgen levels and will be an
55 efficient way to regulate androgen levels (Terrier *et al.* 2016). Rice is a staple food in the Asian
56 region and one of the world's major energy sources. In modern Japanese society, *Oryza sativa* seeds
57 are milled to remove the bran, and the remaining carbohydrate-based endosperm is cooked and
58 consumed. Although rice bran is removed during the milling process, it contains a variety of
59 nutrients that are believed to be useful in maintaining human health (Henderson *et al.* 2012).

60 Tocotrienol is a vitamin E characteristically found in rice bran. The structural difference
61 from tocopherol, a commonly known vitamin E, is the presence of a double bond in the side chain.

62 This structural difference is what makes tocotrienols and tocopherols different in health benefits.
63 Tocotrienols have been reported to have stronger antioxidant and anticancer effects than tocopherols,
64 as well as cardioprotective, antidiabetic, and antiosteoporotic effect (Wong *et al.* 2012).

65 Here, we report the bioactivity of rice bran in increasing androgen secretion from a Leydig
66 cell model, a cell type of the testis that produces androgen hormone, and present tocotrienol as one
67 of its bioactive components.

68

69 **Materials & methods**

70 **Grains and chemicals**

71 Grains were obtained from the commercial markets in Sapporo, Japan. Grains were powdered,
72 immersed in 50% aq. methanol for 24 hr, filtered, and dried to prepare the extract. Commercially
73 available chemicals were purchased from the following companies: erlotinib, H-89, MDL-12,330A,
74 U0126, tocotrienols, and testosterone ELISA kit from Cayman Chemical; Geranylgeraniol from
75 Sigma-Aldrich; Rice bran extract and α-tocotrienol used for the *in vivo* experiments from Phytochem
76 Products Inc. (Sendai, Japan); All others from Fujifilm Wako Pure Chemical Co. (Osaka, Japan).

77

78 **Cell culture**

79 I-10 cells (JCRB9097) were obtained from the Japanese Collection of Research Bioresources Cell
80 Bank (Osaka, Japan). The cells were cultured at 37°C in a 10% CO_2 atmosphere in growth medium
81 [Ham's F-10 medium (Sigma-Aldrich Co. or ThermoFisher Scientific Inc.) supplemented with 10%
82 fetal bovine serum and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL
83 gentamicin)].

84

85 **Testosterone secretion activity assay**

86 I-10 cells were seeded in 48-well plates (2×10^4 cells/well). One day after seeding, the medium was
87 replaced to the sample solution diluted in the growth medium and stimulated for 24 h. The medium
88 was then recovered, and testosterone concentrations were measured with the Testosterone ELISA kit.
89 When inhibitors were used, the cells were pre-treated with an inhibitor for 1 h and then stimulated
90 with the sample in the presence of an inhibitor. Samples and inhibitors were dissolved in water or
91 dimethyl sulfoxide and diluted in growth medium to prepare the sample solution. Dimethyl sulfoxide
92 concentrations in the medium were <0.1%, and the same amount of dimethyl sulfoxide was added to
93 the control cells. Geranylgeraniol (30 μM) was used as the positive control (Ho *et al.* 2016, 2018).
94
95 **Cytotoxicity assay**
96 I-10 cells (1.0×10^4 cells/well) were seeded in 96 well plate and α-tocotrienol (α-T3, 50 μM) was
97 added. After 24 h, cytotoxicity was evaluated by Cytotoxicity LDH Assay Kit-WST (Dojindo
98 Laboratories, Kumamoto, Japan) following the manufacturer's instruction.
99
100 **Intracellular cAMP content**
101 I-10 cells (2.0×10^5 cells/well) were seeded in 6 well plate and α-tocotrienol (α-T3, 50 μM) was
102 added. After 90 min, intracellular cAMP content was evaluated by Cyclic AMP ELISA Kit (Cayman
103 Chemical) following the manufacturer's instruction.
104
105 **Gene expression analysis**
106 I-10 cells were seeded and treated with the samples. After 3 h of treatment, cells were washed with
107 phosphate-buffered saline, and total RNA was extracted using the FastGene RNA Premium Kit
108 (Nippon Genetics Co., Ltd, Tokyo, Japan). Complementary DNA was synthesized from the total
109 RNA using ReverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Gene expression

110 analysis by real-time quantitative polymerase chain reaction was performed with the KAPA SYBR
111 FAST qPCR mix (KAPA Biosystems, Inc.) following general instructions. The primer pairs are listed
112 in Table 1. *Actb* was selected as a reference gene from a preliminary experiment.
113
114 **Western blotting analysis of mitochondria proteins**
115 Mitochondria was isolated from I-10 cells treated with the samples using Mitochondria Isolation Kit
116 for Cultured Cells (ThermoFisher Scientific Inc.). The isolated mitochondria were lysed by sodium
117 dodecyl sulfate sample buffer and heated at 95°C for 5 min. The samples were separated by
118 SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked by 5% non-fat dry
119 milk in Tris-buffered saline containing 0.1% tween-20 for 1 h at room temperature. The following
120 antibodies were used to detect proteins: StAR (Cell Signaling Technology, 1:1000), VDAC (Cell
121 Signaling Technology, 1:2000), anti-rabbit immunoglobulin G horseradish peroxidase-linked
122 antibody (Cell Signalling Technology, 1:2000). Immunostar Zeta (Fujifilm Wako Pure Chemical
123 Co.) was used as the detection reagent. The value of StAR/VDAC was estimated by Image-J
124 software (Schneider, Rasband and Eliceiri 2012).
125
126 **Animal experiments**
127 The experiment was performed with approval (No. 19-0163) from the Institutional Animal Care and
128 Use Committee, Hokkaido University by following National University Corporation Hokkaido
129 University Regulations on Animal Experimentation. Six months old C57BL/6J male mice (n=12)
130 were housed in an air-conditioned room at 23°C±2°C with a light period from 8:00 am to 8:00 pm.
131 The mice had free access to their diet and water. The mice were grouped to control and sample
132 treating group (1st trial: rice bran extract; 2nd trial α-tocotrienol) and fed with MF diet (Oriental Yeast
133 Co., ltd., Tokyo, Japan) or MF diet supplemented with 5% of rice bran extract (6.6% α-tocotrienol,

134 16.6% γ-tocotrienol and 25.2% tocopherols) or 0.1% α-tocotrienol (70.8% purity, contains 0.9%
135 tocopherols) for two weeks. The mice were anesthetized by isoflurane, sacrificed and the blood and
136 testes were collected. Blood was placed for 30 min, centrifuged for 10 min at 1500g, and the serum
137 was recovered. The testosterone in the serum was measured with the Testosterone ELISA kit. The
138 testes were washed with phosphate buffered saline and immersed in Ham's F-10 medium. After 2 h,
139 the medium was collected, and testosterone concentration was evaluated.
140

141 **Statistical analysis**

142 Experiments were repeated at least twice, and representative data are expressed as mean ± standard
143 deviation (SD). Data were analyzed by GraphPad Prism software (ver.10) with the statistical
144 methods indicated in the figure legends.
145

146 **Results**

147 **Extract of unpolished rice increases testosterone secretion from Leydig cells**

148 Since preliminary experiment showed that extract of unpolished rice increased testosterone
149 secretion from I-10 cells, a Leydig cell model, we evaluated the activity of various grain extracts.
150 Geranylgeraniol (GG) was used as positive control in the experiment (Ho *et al.* 2016, 2018). Extracts
151 of unpolished white rice (Koshihikari) increased testosterone secretion 2.3-fold, while polished rice
152 had no effect (Figure 1A). Among the other tested rice varieties, red rice showed a slight increase,
153 while green rice (Akunemochi) showed the highest value with a 2.8-fold increase. Extracts of other
154 grains also showed activity to increase the testosterone secretion from I-10 cells, and the same trend
155 was observed that the activity decreased when the bran part was removed (Figure 1B). Therefore, we
156 focused on the components contained in rice bran.
157

158 **Tocotrienol enhances testosterone secretion from Leydig cells**

159 Components in rice bran were selected (Figure 2a) (Henderson *et al.* 2012), and evaluated
160 for their activity in stimulating testosterone secretion from I-10 cells (Figure 2b). α-Tocotrienol
161 (α-T3) was the only active component, showing concentration dependent activity between 10-50 μM
162 (Figure 2c). α-T3 was not cytotoxic to I-10 cells at 50 μM (Figure S1). The isomers of T3 were also
163 tested and γ-T3 exhibited the activity, and β-T3 and δ-T3 had no activity (Figure 2d). The T3 content
164 in the unpolished white rice extract was analyzed, but sufficient levels of T3 to stimulate I-10 cells
165 were not detected (Figure S2), however, we focused on α-T3 since it is the characteristic vitamin E
166 in the rice bran.

167

168 **Steroidogenic acute regulatory protein is responsible for the activity of α-T3**

169 To explore the molecular mechanism of α-T3 to enhance testosterone secretion from I-10
170 cells, change in gene expression related to the testosterone biosynthesis was analyzed. Gene
171 expression of steroidogenic acute regulatory protein (StAR) was 2.4 times upregulated following the
172 stimulation by α-T3 and no significant difference was observed for other genes (Figure 3a).

173 StAR is a protein responsible for transporting cholesterol from the outer to the inner
174 mitochondrial membrane. This step is a rate limiting step for the biosynthesis of steroid hormones
175 and thus the enhanced expression of *Star* is expected to contribute to the activity of α-T3.

176 Expression of *Star* is regulated through cyclic AMP/protein kinase A (PKA) pathway
177 (Manna *et al.* 2009). To evaluate if this pathway is involved in the mechanism of α-T3, PKA
178 inhibitor H-89 or adenylyl cyclase inhibitor MDL10,330A were co-incubated with α-T3. These two
179 inhibitors decreased the testosterone secretion stimulated by α-T3 (Figure 3b,c). Intracellular cAMP
180 level after α-T3 stimulation also showed a significant increase (Figure 3d), indicating the
181 involvement of cAMP/PKA pathway in the activity of α-T3.

182 In addition to the upregulation of *Star* expression, PKA also rapidly enhances testosterone
183 synthesis through epidermal growth factor receptor (EGFR) and MAPK mediated translocation of
184 StAR to the mitochondria (Evaul *et al.* 2008). After co-incubation of α-T3 with EGFR inhibitor
185 erlotinib or MAPK inhibitor U0126, progesterone released in the medium was evaluated. To avoid
186 the influence of elevated *Star* expression, short term effect of α-T3 on this pathway was evaluated.
187 Progesterone was selected since it was released from the cell at the higher level than testosterone and
188 was easier to detect the change in short term trial. Addition of erlotinib or U0126 completely
189 diminished the activity of α-T3 (Figure 3e,f), but these two inhibitors also reduced the basal level of
190 steroid hormone release from I-10 cells, suggesting the importance of this pathway and remaining a
191 question if α-T3 activates this pathway. Thus, translocation of StAR to the mitochondria was also
192 evaluated. Mitochondria was isolated from the α-T3 stimulated I-10 cells and the amount of StAR
193 was compared with control by western blotting (Figure 3g and Figure S3). α-T3 stimulated I-10 cells
194 showed significant increase in mitochondria localized StAR suggesting that α-T3 not only enhance
195 the gene expression of *Star* but also induced its translocation to enhance steroid hormone synthesis.
196
197 **Preliminary results of the effects of rice bran extract on mice**
198 To evaluate the *in vivo* effects of rice bran extract, mice were fed a diet supplemented with
199 5% rice bran extract for 2 weeks and serum testosterone levels as well as testosterone secretion from
200 isolated testes were evaluated. Result showed no difference between control mice and mice fed rice
201 bran diet (Figure 4a). The rice bran extract employed in this experiment contained 23.2% of T3
202 (α:6.6%, γ: 16.6%) and 25.2% of tocopherol. Thus, diet supplemented with 0.1% of α-T3, containing
203 less than 1% tocopherol, was fed to mice and serum testosterone level were examined. The result
204 showed a trend to increase the serum testosterone level in α-T3 fed mice (Figure 4b). No significant
205 difference in food intake (control 3.6±0.5; α-T3 3.9±0.5 g/day/mouse) and body weight change

206 (control +0.42; α-T3 −0.38 g) was observed.

207

208 **Discussion**

209 Decreased testosterone levels are caused by stress and aging, and supporting these levels
210 with food will be an alternative to medication. We have shown that various grains have the ability to
211 stimulate steroid hormone secretion from Leydig cells, but this effect was reduced when the bran
212 portion was removed (Figure 1b). Although the result is at the cellular level, it gives a suggestion
213 that consuming unpolished grains may protect from the testosterone deficiency.

214 Among the rice bran components, T3 was found as a bioactive component that enhances
215 testosterone secretion from Leydig cells, with α-T3 showing the highest activity (Figure 2d). Wheat
216 (Babura *et al.* 2017; Lachman *et al.* 2018), barley (Babura *et al.* 2017; Lachman *et al.* 2018), adlay
217 (Babura *et al.* 2017), and millet (Ji *et al.* 2019) also contain T3 suggesting their contribution in the
218 activity. However, the T3 content in each grain varies, indicating the presence of other bioactive
219 components.

220 T3 shows multiple bioactivities, but the current activity is differentially selective for the
221 T3 family (α, β, γ, δ). Antioxidant activity is not affected by the position or number of methyl groups
222 (Müller *et al.* 2010), anti-cancer activity favors γ-T3 and δ-T3 over α-T3 (Wu *et al.* 2010), and
223 suppression of HMG-CoA reductase, which is associated with cardioprotective effects, is more
224 potent for γ-T3 than with α-T3 (Parker *et al.* 1993). These differences suggested a unique
225 mechanism for α-T3 to enhance testosterone secretion. Note that we did not evaluate the uptake of
226 T3 family by I-10 cells, or their stability in the medium. Thus, differences in uptake and stability
227 may contribute to differences in activity of T3 family, just as in the case of vitamin K1 and
228 menaquinone-4, which differences in cellular uptake have been implicated in their ability to promote
229 testosterone production in I-10 cells (Ito *et al.* 2011).

230 The above suggestion is clearly shown from the mechanistic analysis, that α-T3 enhance
231 steroid hormone synthesis through StAR, which gene expression and activation is regulated by PKA
232 (Manna *et al.* 2009). α-T3 likely use PKA pathway, supported by the fact that adenylyl cyclase
233 inhibitor and PKA inhibitor diminishes the activity of α-T3 (Figure 3bc). The anticancer activity of
234 γ-T3 is due to the suppression of NF-κB through PI3K/AKT pathway (Alawin *et al.* 2016). This
235 pathway contributes to the anti-inflammatory properties of γ-T3 and may be related to its
236 cardioprotective effects. Thus, the mechanism of testosterone secretion enhancement by α-T3 differ
237 from other reported bioactivities. One concern is that although activation of the PKA pathway by
238 cAMP agonists induces expression of *Cyp11a1*, *Cyp17a1* and *Hsd3b*, no increase was observed by
239 α-T3 (Figure 3a) (Kempná *et al.* 2010; King 2012). One explanation for this is that the response of
240 those genes to cAMP is slower than *Star* (Lejeune *et al.* 1998; King 2012), and thus no upregulation
241 was observed in our experiment.

242 We were unable to identify a direct target protein of α-T3, but two possibilities can be
243 raised. The analysis of γ-T3 on HER2-positive human breast cancer cells showed an accumulation of
244 γ-T3 in lipid rafts and disruption of microdomains (Alawin *et al.* 2016). Disruption of lipid
245 microdomain has been reported to causes an increase in Gs signaling (Allen *et al.* 2009). Since
246 signaling of Gs leads to the activation of PKA, this mechanism is one possible explanation for the
247 enhancement of testosterone secretion by α-T3. Another possibility is an estrogen receptor signaling.
248 T3 can bind to estrogen receptor β (Nakaso *et al.* 2014). Intracellular estrogen receptors are not
249 involved in the regulation of testosterone synthesis, suggested from the co-incubation of antagonist
250 with α-T3 (data not shown). However, G-protein coupled estrogen receptor 1 (GPER1) located at the
251 plasma membrane regulates steroid hormone secretion from Leydig cell (Gorowska-Wojtowicz *et al.*
252 2018), and may function as the targets of α-T3.

253 Preliminary test with C57BL6/J mice showed an increasing trend with purified α-T3 intake,

but not with rice bran extract. Several studies showing beneficial effects of T3 have used highly purified T3 (Tsuduki *et al.* 2013; Wong *et al.* 2017). When crude extracts were used, they contained more than twice as much T3 as tocopherol (Yamada *et al.* 2008). It has been reported that the presence of α-tocopherol in the diet together with α-T3 decreases α-T3 concentrations in the plasma and tissues of rats fed the diet (Ikeda *et al.* 2003). Thus, it is presumed that the mice fed rice bran extract did not reach sufficient T3 levels to show an effect because of the presence of approximately equal amounts of T3 and tocopherol in the diet.

We conducted only preliminary *in vivo* studies because our goal was to use rice bran as a food for support and prevention of LOH patients, and the result suggested that simple consumption of rice bran might be ineffective. However, perhaps due to the influence of social dominance (Machida *et al.* 1981), the testosterone levels of the mice in the two experiments employing rice bran extract or α-T3 were largely different. Therefore, further studies are needed to assess the effectiveness of rice bran and α-T3.

Conclusions

α-T3 is a characteristic vitamin E found in rice bran with variety of health benefits. The finding that α-T3 enhances the testosterone secretion from Leydig cells through activation and enhanced gene expression of StAR adds another benefit of T3. Preliminary *in vivo* experiments suggests that consumption of purified α-T3 may be effective in increasing testosterone content in the body. However, it has also been suggested that the presence of tocopherol may interfere with this effect. Given that rice bran contains both tocopherols and T3 (Sookwong *et al.* 2007), selecting a strain of rice with a higher ratio of T3 to tocopherol may be effective approach to achieve the goal.

Supplementary material

Supplementary material is available at Bioscience, Biotechnology, and Biochemistry online.

Data Availability

The data underlying this article are available in the article.

Author contribution

Investigation- NT, TN, HK, and YA; Formal analysis- NT and EK; Conceptualization, Funding Acquisition, and Writing Original Draft- EK. Review & Editing is done by all the authors.

Disclosure statement

Authors declare no conflict of interests.

Funding

This work was supported by the The Public Foundation of Elizabeth Arnold－Fuji.

References

Alawin OA, Ahmed RA, Ibrahim BA *et al.* Antiproliferative effects of γ-tocotrienol are associated with lipid raft disruption in HER2-positive human breast cancer cells. *J Nutr Biochem* 2016;**27**:266–77.

Allen JA, Yu JZ, Dave RH *et al.* Caveolin-1 and Lipid Microdomains Regulate G s Trafficking and Attenuate G s /Adenylyl Cyclase Signaling. *Mol Pharmacol* 2009;**76**:1082–93.

Babura SR, Abdullah SNA, Khaza'ai H. Advances in Genetic Improvement for Tocotrienol Production: A Review. *J Nutr Sci Vitaminol (Tokyo)* 2017;**63**:215–21.

Evaul K, Hammes SR. Cross-talk between G Protein-coupled and Epidermal Growth Factor Receptors Regulates Gonadotropin-mediated Steroidogenesis in Leydig Cells. *J Biol Chem*

302 2008;**283**:27525–33.

303 Gianatti EJ, Grossmann M. Testosterone deficiency in men with Type 2 diabetes: pathophysiology and

304 treatment. *Diabet Med* 2020;**37**:174–86.

305 Gorowska-Wojtowicz E, Dutka P, Kudrycka M *et al.* Regulation of steroidogenic function of mouse

306 Leydig cells: G-coupled membrane estrogen receptor and peroxisome proliferator-activated

307 receptor partnership. *J Physiol Pharmacol* 2018;**69**:373–90.

308 Habito RC, Montalto J, Leslie E *et al.* Effects of replacing meat with soyabean in the diet on sex hormone

309 concentrations in healthy adult males. *Br J Nutr* 2000;**84**:557–63.

310 Henderson AJ, Ollila CA, Kumar A *et al.* Chemopreventive Properties of Dietary Rice Bran: Current

311 Status and Future Prospects. *Adv Nutr* 2012;**3**:643–53.

312 Ho HJ, Shirakawa H, Giriwono PE *et al.* A novel function of geranylgeraniol in regulating testosterone

313 production. *Biosci Biotechnol Biochem* 2018;**82**:956–62.

314 Ho HJ, Shirakawa H, Yoshida R *et al.* Geranylgeraniol enhances testosterone production via the

315 cAMP/protein kinase A pathway in testis-derived I-10 tumor cells. *Biosci Biotechnol Biochem*

316 2016;**80**:791–7.

317 Huhtaniemi I, Forti G. Male late-onset hypogonadism: pathogenesis, diagnosis and treatment. *Nat Rev*

318 *Urol* 2011;**8**:335–44.

319 Ikeda S, Tohyama T, Yamashita K *et al.* Dietary α-Tocopherol Decreases α-Tocotrienol but Not

320 γ-Tocotrienol Concentration in Rats. *J Nutr* 2003;**133**:428–34.

321 Ito A, Shirakawa H, Takumi N *et al.* Menaquinone-4 enhances testosterone production in rats and

322 testis-derived tumor cells. *Lipids Health Dis* 2011;**10**:158.

323 Ji J, Liu Y, Ge Z *et al.* Oleochemical Properties for Different Fractions of Foxtail Millet Bran. *J Oleo Sci*

324 2019;**68**:709–18.

325 Kempná P, Hirsch A, Hofer G *et al.* Impact of Differential P450c17 Phosphorylation by cAMP

326 Stimulation and by Starvation Conditions on Enzyme Activities and Androgen Production in
327 NCI-H295R Cells. *Endocrinology* 2010;**151**:3686–96.
328 King SR. Gonadal transactivation of STARD1, CYP11A1 and HSD3B. *Front Biosci* 2012;**17**:824.
329 Kloner RA, Carson C, Dobs A *et al.* Testosterone and Cardiovascular Disease. *J Am Coll Cardiol*
330 2016;**67**:545–57.
331 Lachman J, Hejtmánková A, Orsák M *et al.* Tocotrienols and tocopherols in colored-grain wheat,
332 tritordeum and barley. *Food Chem* 2018;**240**:725–35.
333 Lejeune H, Sanchez P, Chuzel F *et al.* Time-course effects of human recombinant luteinizing hormone on
334 porcine Leydig cell specific differentiated functions. *Mol Cell Endocrinol* 1998;**144**:59–69.
335 Machida T, Yonezawa Y, Noumura T. Age-associated changes in plasma testosterone levels in male mice
336 and their relation to social dominance or subordinance. *Horm Behav* 1981;**15**:238–45.
337 Manna PR, Dyson MT, Stocco DM. Regulation of the steroidogenic acute regulatory protein gene
338 expression: present and future perspectives. *Mol Hum Reprod* 2009;**15**:321–33.
339 Matousek RH, Sherwin BB. Sex steroid hormones and cognitive functioning in healthy, older men. *Horm*
340 *Behav* 2010;**57**:352–9.
341 Müller L, Theile K, Böhm V. In vitro antioxidant activity of tocopherols and tocotrienols and comparison
342 of vitamin E concentration and lipophilic antioxidant capacity in human plasma. *Mol Nutr Food*
343 *Res* 2010;**54**:731–42.
344 Nakaso K, Tajima N, Horikoshi Y *et al.* The estrogen receptor β-PI3K/Akt pathway mediates the
345 cytoprotective effects of tocotrienol in a cellular Parkinson's disease model. *Biochim Biophys Acta -*
346 *Mol Basis Dis* 2014;**1842**:1303–12.
347 Parker RA, Pearce BC, Clark RW *et al.* Tocotrienols regulate cholesterol production in mammalian cells
348 by post- transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Biol*
349 *Chem* 1993;**268**:11230–8.

350 Rodrigues dos Santos M, Bhasin S. Benefits and Risks of Testosterone Treatment in Men with
351 Age-Related Decline in Testosterone. *Annu Rev Med* 2021;**72**:75–91.
352 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat*
353 *Methods* 2012;**9**:671–5.
354 Sookwong P, Nakagawa K, Murata K *et al.* Quantitation of Tocotrienol and Tocopherol in Various Rice
355 Brans. *J Agric Food Chem* 2007;**55**:461–6.
356 Terrier J-E, Isidori AM. How Food Intakes Modify Testosterone Level. *J Sex Med* 2016;**13**:1292–6.
357 Tsuduki T, Kuriyama K, Nakagawa K *et al.* Tocotrienol (Unsaturated Vitamin E) Suppresses
358 Degranulation of Mast Cells and Reduces Allergic Dermatitis in Mice. *J Oleo Sci* 2013;**62**:825–34.
359 Wong RSY, Radhakrishnan AK. Tocotrienol research: past into present. *Nutr Rev* 2012;**70**:483–90.
360 Wong W-Y, Ward LC, Fong CW *et al.* Anti-inflammatory γ- and δ-tocotrienols improve cardiovascular,
361 liver and metabolic function in diet-induced obese rats. *Eur J Nutr* 2017;**56**:133–50.
362 Wu S-J, Ng L-T. Tocotrienols Inhibited Growth and Induced Apoptosis in Human HeLa Cells Through
363 the Cell Cycle Signaling Pathway. *Integr Cancer Ther* 2010;**9**:66–72.
364 Yamada Y, Obayashi M, Ishikawa T *et al.* Dietary tocotrienol reduces UVB-induced skin damage and
365 sesamin enhances tocotrienol effects in hairless mice. *J Nutr Sci Vitaminol (Tokyo)*
366 2008;**54**:117–23.
367
368

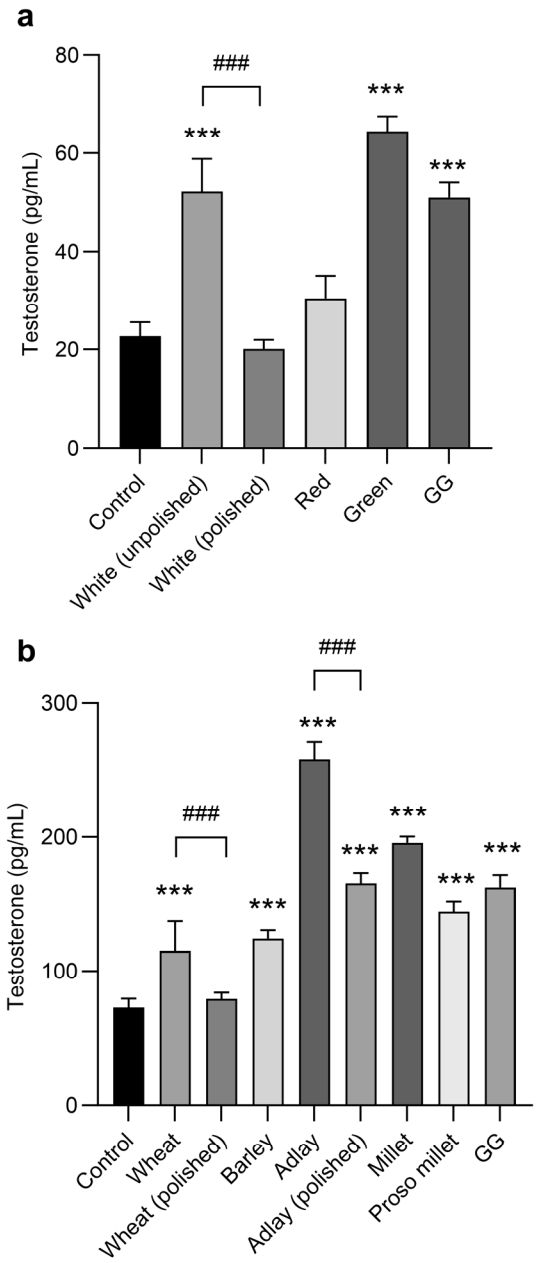


Figure 1. Extract of various grains enhance testosterone secretion from I-10 cells. Extracts of each grain (1 mg/mL) were added to the medium of I-10 cells, incubated for 24 h, and the testosterone secreted into the medium was quantified by ELISA. a) Differences in rice varieties. b) Differences in grain varieties. Geranylgeraniol (GG, 30 μM) was used as positive control. ***$p<0.001$ vs control, ###$p<0.001$ between the indicated bars (n=3, Tukey's test).

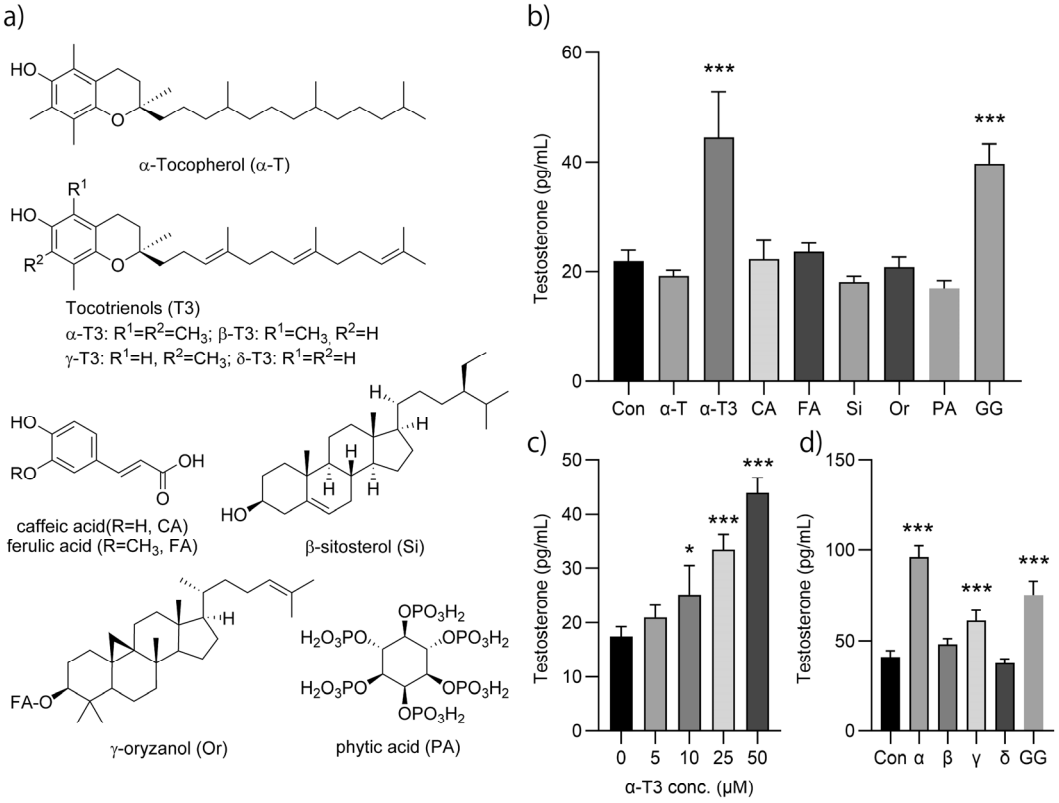


Figure 2. Testosterone secretion of I-10 cells after stimulation by the components of rice bran. a) structures of tested components. b) I-10 cells are stimulated by 50 μM of components for 24 h and the testosterone concentration in the medium was quantified by ELISA. c) Concentration dependency of α-T3 to stimulate testosterone secretion. d) Isomers of T3 were tested at 50 μM for testosterone secretion. Geranylgeraniol (GG, 30 μM) was used as a positive control. ***$p<0.001$, *$p<0.05$ vs control (n=3, Tukey's test).

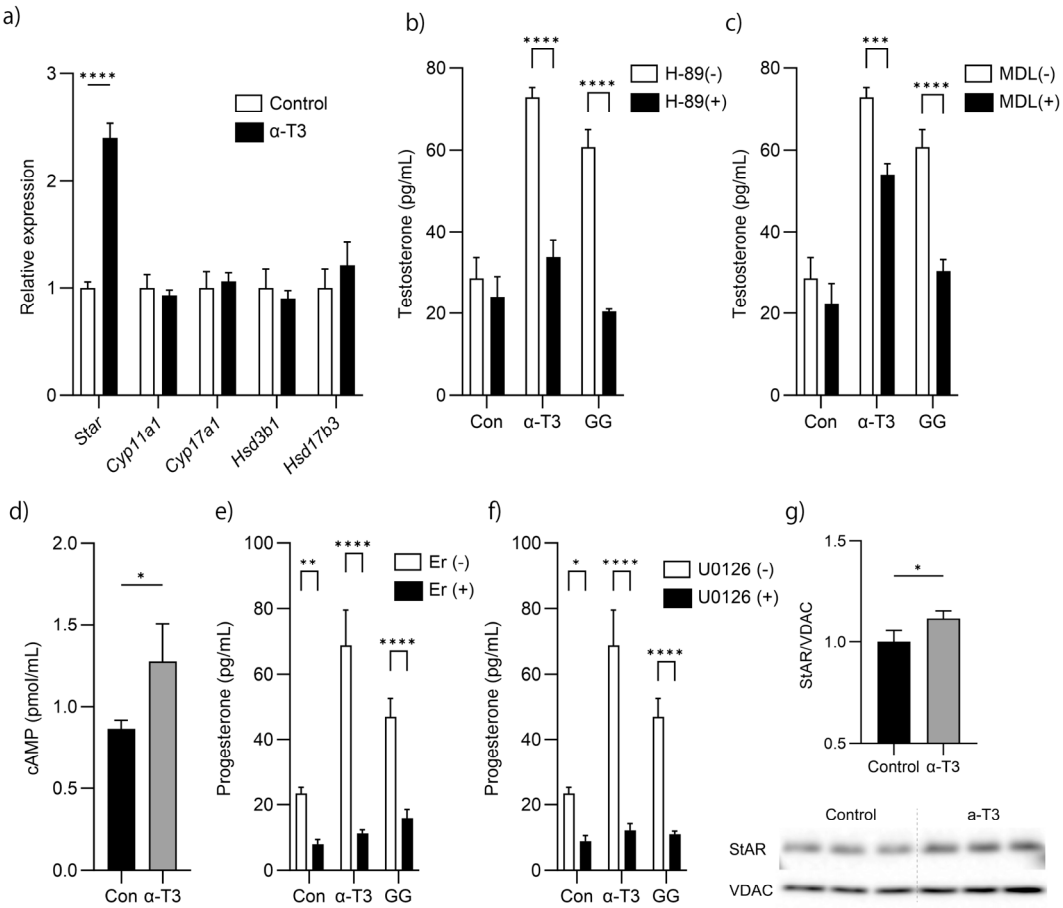


Figure 3. Pathway analysis of the α-T3 stimulated testosterone secretion in I-10 cells. a) Analysis of testosterone biosynthesis genes. b,c) Co-incubation of α-T3 (50 μM) with H-89 (PKA inhibitor, 10 μM), and MDL12,330A (adenylyl cyclase inhibitor, 10 μM). ****p<0.0001, ***p<0.001 (n=3, Tukey's test). d) Intracellular cAMP level after stimulation by α-T3 (n=4, t-test). e,f) Co-incubation of α-T3 (50 μM) with Erlotinib (EGFR inhibitor, 5 μM), and U0126 (MAPK inhibitor, 20 μM). ****p<0.0001, **p<0.01, *p<0.05 (n=3, Tukey's test). g) Western blotting analysis of mitochondrial StAR protein. *p<0.05 (n=3, t-test).

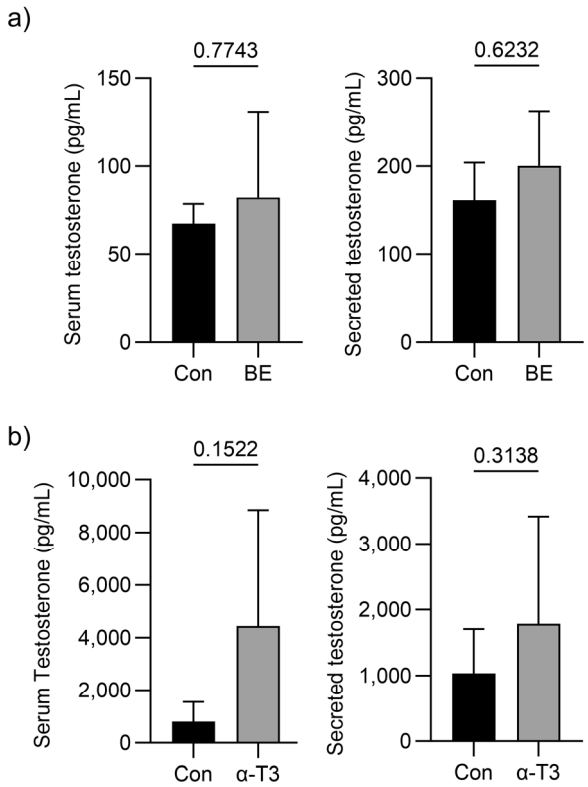


Figure 4. Serum testosterone level and testosterone level secreted from the isolated testis in mouse fed a) rice bran extract (BE) or b) α-tocotrienol (α-T3) for 2 weeks. (n=4, t-test).

395

396 Table 1. Primer pairs used for qPCR analysis

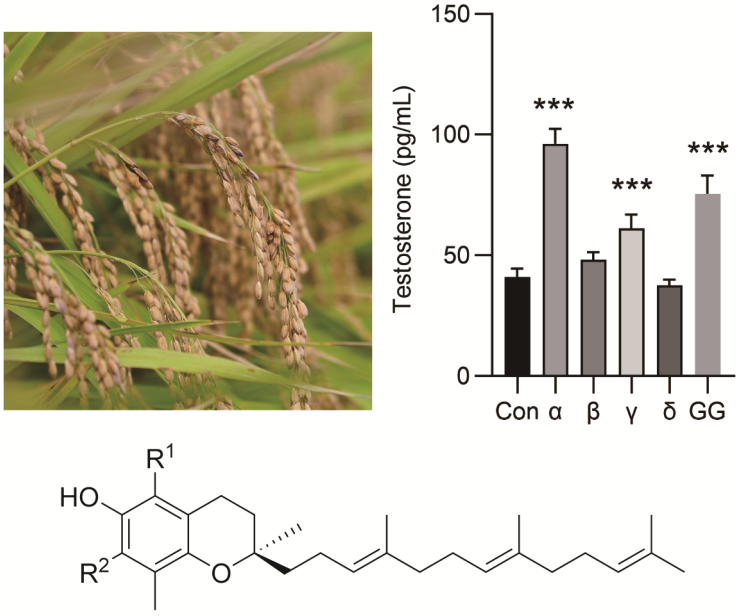
Target gene	Forward	Reverse
Star	TGGAAAAGACACGGTCATCA	CTCCGGCATCTCCCCAAAAT
Cyp11a1	CGTGACCAGAAAAGACAACA	AGGATGAAGGAGAGGAGAGC
Cyp17a1	TGGGCACTGCATCACGATAA	GCTCCGAAGGGCAAATAACT
Hsd3b1	AGTGATGGAAAAAGGGCAGGT	GCAAGTTTGTGAGTGGGTTAG
Hsd17b3	AACGCAACATCAGCAACAGA	CAGCCCCACCTCACCCTACC
Actb	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA

397

398

Graphical Abstract Caption

Tocotrienol, a constituent of rice bran, was discovered as a bioactive compound that promotes steroidogenesis in Leydig cells.



Tocotrienols (T3)α-T3: R^1=R^2=CH_3; β-T3: R^1=CH_3, R^2=H; γ-T3: R^1=H, R^2=CH_3; δ-T3: R^1=R^2=H