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Synthesis of nandrolone in the human ovary

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Introduction

Recent studies have revealed that low concentrations of urinary norandrosterone (3α-hydroxy-5α-estran-17-one) of possible endogenous origin can be detected in the urine of athletes [1-7]. Human in vivo production of nandrolone (17β-hydroxy-estr-4-ene-3-one) has been reported in the presence of aromatase-rich tissue in the ovarian follicle at the time of ovulation [8]. During pregnancy nandrolone has been detected in plasma [9], as well as its metabolites in urine [10]. A study of urinary concentrations of norandrosterone during one menstrual cycle in 12 female volunteers showed a clear maximum at the time of ovulation [11]. Endogenous nandrolone seems to be produced as a by-product of the enzymatic conversion of androgens to estrogens as shown in porcine granulosa cells [12], equine testis [13] and equine and human placenta [14].

The objective of the present study was to study the aromatisation reaction for the conversion of androgens to estrogens in order to find out whether 19-norandrogens (nandrolone or norandrostenedione (estr-4-ene-3,17-dione)) are formed as a by-products of the aromatase activity or by different synthesis routes. We therefore studied excretion of 19-norandrogens and estrogens from granulosa cell cultures *in vitro* after stimulation and inhibition of the aromatase enzyme system. A simplified illustration of the enzyme system for the conversion of androgens to estrogens is presented in Figure 1.
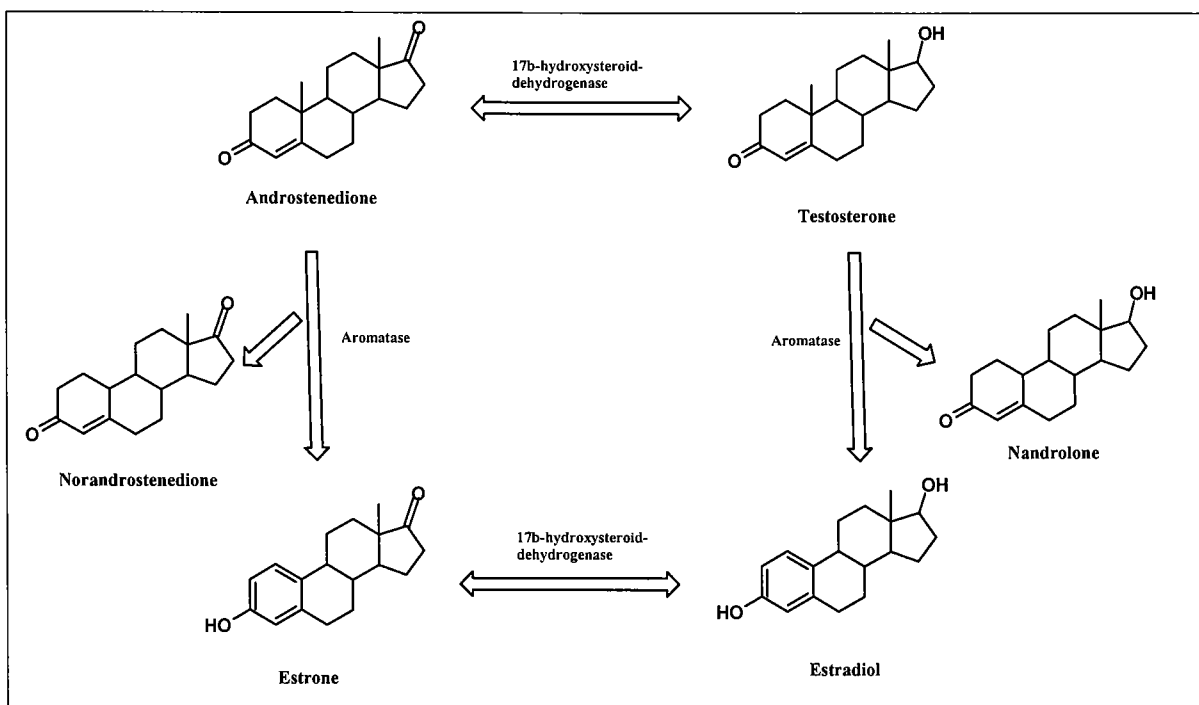
17b-hydroxysteroid-dehydrogenase

Androstenedione

Testosterone

Aromatase

Aromatase

Norandrostenedione

Nandrolone

17b-hydroxysteroid-dehydrogenase

Estrone

Estradiol

Figure 1. A simplified illustration of the enzyme system for the conversion of androgens to estrogens.

Materials and methods

Cell cultures

The model matrix in this work were granulosa-lutein cells obtained from follicular fluid. Granulosa-lutein cells were obtained from women undergoing in vitro fertilisation. Follicular fluid from stimulated cycles is an excellent source of estrogen producing granulosa cells as the aromatase activity is highest at ovulation. The granulosa-lutein cells were purified, isolated and cultivated using the methods established by Fedorcsàk et al [15]. The cells were incubated in the presence of testosterone with and without aromatase inhibitor and product inhibitor. The granulosa cells in culture produced steroids that were excreted into the culture medium. After incubation for 24 hours, the culture medium was removed from each well and stored at –20 °C until analysis. GC-MS was utilised for the determination of nandrolone, norandrostenedione, androstenedione (androst-4-ene-3,17-dione), testosterone, estradiol, estrone and progesterone in the granulosa cell cultures.

Analytical sample preparation

All reagents and solvents were of analytical grade quality and purchased by Merck (Darmstadt, Germany). Nandrolone, testosterone, norandrostenedione, androstenedione, estrone, estradiol, progesterone, 4-hydroxy-androstenedione and methyltestosterone were obtained from Sigma (St. Louis, MO, USA). MSTFA (*N*-Methyl-*N*-

(trimethylsilyl)trifluoroacetamide) was supplied by Pierce (Toronto, Canada), ammonium iodide from Sigma and etanthiol from Merck.

The method used for culture medium sample preparation was modified from routine sample clean-up for anabolic-androgenic steroids applied in the IOC accredited laboratories. This procedure goes back to the work of Donike et al [16] and has been described before [17,18]. The volume of culture medium was dependent on the amount of cells available, usually between 0.5 to 2.5 ml. To culture medium in a glass tube, 0.8 ml of Tris(hydroxymethyl)-aminometane buffer (pH 8-8.3) and 100 ng internal standard, methyltestosterone, were added. The samples were extracted with 5 ml n-pentane while shaking for 10 minutes and then centrifuged at 2000 g for 10 minutes. The aqueous phase was frozen in a bath with dry ice in acetone and the organic phase was collected and evaporated to dryness. The dry residue was derivatised with 40 µl MSTFA/NH$_4$I/ethanthiol (1000:2:6, v/w/w) for 20 minutes at 70 °C.

GC-MS

The GC-MS analyses were performed on an Agilent 5973N mass spectrometer coupled to an Agilent 6890 gas chromatograph with automatic sampler Agilent 7683, all from Agilent (Palo Alto, CA, USA). The column was a fused silica cross-linked methylsilicon Ultra 1 (Agilent) (17 mx0.2 mm I.D., film thickness 0.11 mm). Helium carrier gas at a linear velocity of 40 cm/s was used. Oven temperature was programmed as follows: 100 °C, held for 1 minute, raised 40 °C/min to 220 °C, then 2 °C/min to 235 °C, then 40 °C/min to final temperature 310 °C and held for 1 minute. Injected volume was 1 µl in splitless mode. Injector and transfer line temperature were 280 °C. The mass spectrometer was operated in electron impact ionisation mode at 70 eV. The following characteristsic ions were monitored in selected ion monitoring (SIM) acquisition mode: Group 1: 2-9.25 min, m/z 194.1, 309.2, 325.2, 399.2, 401.3, 403.3, 414.3, 415.3, 416.3, 418.3, 430.3, group 2: 9.25-10 min, m/z 209.1, 285.2, 309.2, 325.2, 399.3, 401.3, 414.3, 415.3, 416.3, 417.3, 430.3, 432.3, group 3: 10-16.4 min, m/z 157.1, 301.2, 443.3, 446.3, 458.3. Dwell time was 50 ms.

Results and Discussion

Validation of analytical method

The selectivity of the method was studied by analysing different cell culture media ($n=4$). No interfering peaks were observed for any of the compounds assayed. Results from validation of the analytical method are presented in Table 1. Recovery results for the different analytes were determined to be higher than 80 %, except – as expected – for estradiol.

Table 1. Results from validation of analytical method.

Compound	LOD [a] ng/ml	LOQ [b] ng/ml	Intra-assay precision (CV %) [c]	Inter-assay precision (CV %) [c]	Accuracy (%) [d]
Androstenedione	0.06	0.19	3.5	6.3	-2.3
Testosterone	0.09	0.30	10	17	0.4
Norandrostenedione	0.08	0.20	1.1	8.5	-3.0
Nandrolone	0.05	0.17	5.3	24	2.0
Estradiol	0.16	0.50	4.9	36	8.9
Estrone	0.16	0.50	3.9	26	3.3
Progesterone	0.30	1.80	3.5	9.7	-2.9

[a] LOD = limit of detection
[b] LOQ = limit of quantitation
[c] Intra- and inter-assay precision data from 0.4 ng/ml samples, except for estradiol at 2 ng/ml
[d] Accuracy data from 2 ng/ml samples

The performance criteria for nandrolone were satisfactory. LOD for progesterone is higher than for the other compounds. Progesterone was measured qualitatively to test viability of cell cultures.

Granulosa-lutein cell cultures

The cultured cells produced steroids that were excreted into the culture medium. Limiting factors are the volume of follicular fluid (24 wells à 500 µl) and the number of cells in culture (minimum 50 000 cells in each well). Viability was tested by Trypan Blue exclusion and progesterone production (measured by GC-MS). Viability in the established cell cultures was always above 90 %. All cell cultures produced progesterone, the mean concentration of progesterone was 4.5 pmol per 1000 cells.

19-norandrogen production in cell cultures without substrate added

The concentration of steroids in the culture medium was measured without stimulation in order to investigate the endogenous production of 19-norandrogens in the cell cultures. Very few cell cultures produced 19-norandrogens above the detection limit without stimulation. In 2 of 29 cell cultures the production rate of nandrolone was sufficient to give concentrations above the detection limit. The highest concentration was 0.3 ng/ml. The concentration of norandrostenedione was above the detection limit in 4 of 29 cell cultures. This demonstrates that granulosa cell cultures can produce 19-norandrogens without stimulation.

Nandrolone production in cell cultures stimulated with testosterone

Granulosa cell cultures from 10 patients were stimulated by adding testosterone as substrate. Culture media were fortified with testosterone in increasing concentrations of 20, 200, 2000 nmol/l. Increased testosterone concentration led to increased nandrolone production in 7 of 10 cell cultures. A typical example of stimulation of nandrolone production is presented in Figure 2. This demonstrates that testosterone is a good substrate for nandrolone production in granulosa cell cultures. The nandrolone production is considerable at 200 nmol/l. and there is only a minor increase in nandrolone concentration from increasing the testosterone concentration from 200 to 2000 nmol/l. From this we assume that the enzyme system is saturated and that zero-order kinetics exist at 2000 nmol/l testosterone. The concentration 2000 nmol/l testosterone was used in the rest of the experiments.
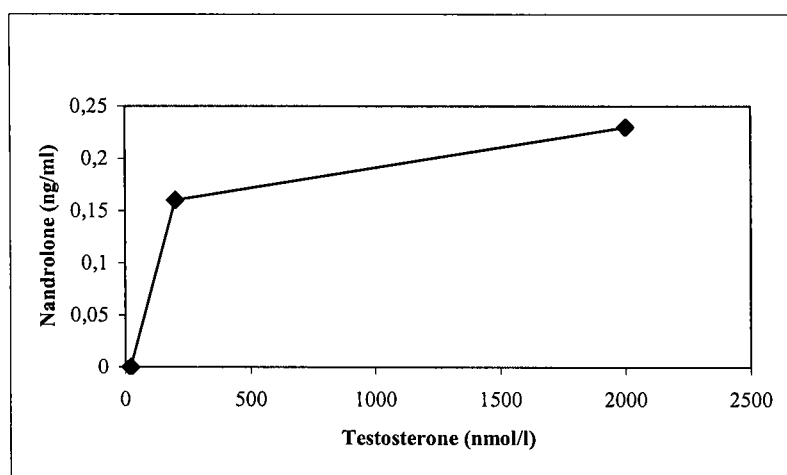

Figure 2. Nandrolone production in granulosa cell cultures as a function of testosterone stimulation.

Inhibition with aromatase inhibitor 4-OH-Androstenedione

The effect of aromatase inhibitor 4-hydroxy-androstenedione (4-OHA) was studied by comparing the steroid production of the granulosa cell cultures where testosterone (2000 nmol/l) was added as a substrate, with the steroid production from granulosa cell cultures with substrate and aromatase inhibitor 4-OHA (1000 nmol/l). Additionally a set of control samples with only 4-OHA (1000 nmol/l) was investigated. Granulosa cell cultures from 13 patients were studied.

4-OHA is a type I competitive inhibitor with a high affinity for the aromatase, the effect comes from an irreversible binding to the aromatase apoprotein [19].

All cell cultures produced nandrolone after stimulation with testosterone. The cell cultures produced approximately 100 times more estradiol (and 10 times more estrone and androstenedione) than nandrolone. In Figure 3 the concentration of nandrolone is plotted against the concentration of estradiol produced by the granulosa cell cultures, where testosterone was added as substrate. The concentrations correlate very well. This is an indication that the production of nandrolone and estradiol are catalysed by the same enzyme.
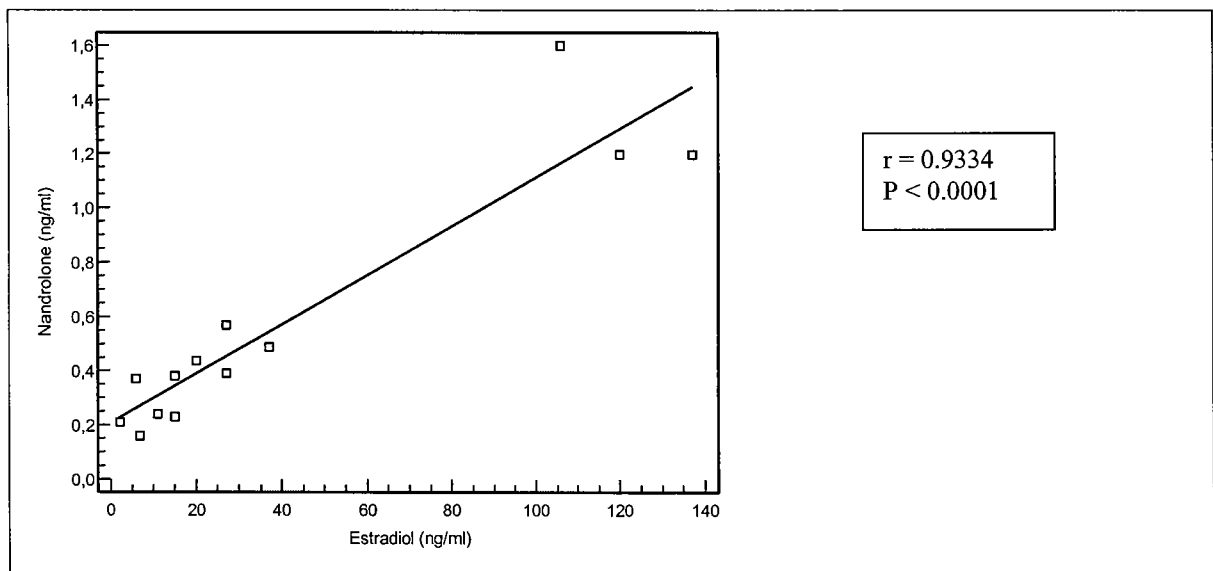

Figure 3. Correlation between nandrolone and estradiol concentrations in granulosa cell cultures stimulated with testosterone.

The effect of the inhibition with 4-OHA on nandrolone and estradiol production is presented in Figure 4 and Figure 5, respectively.
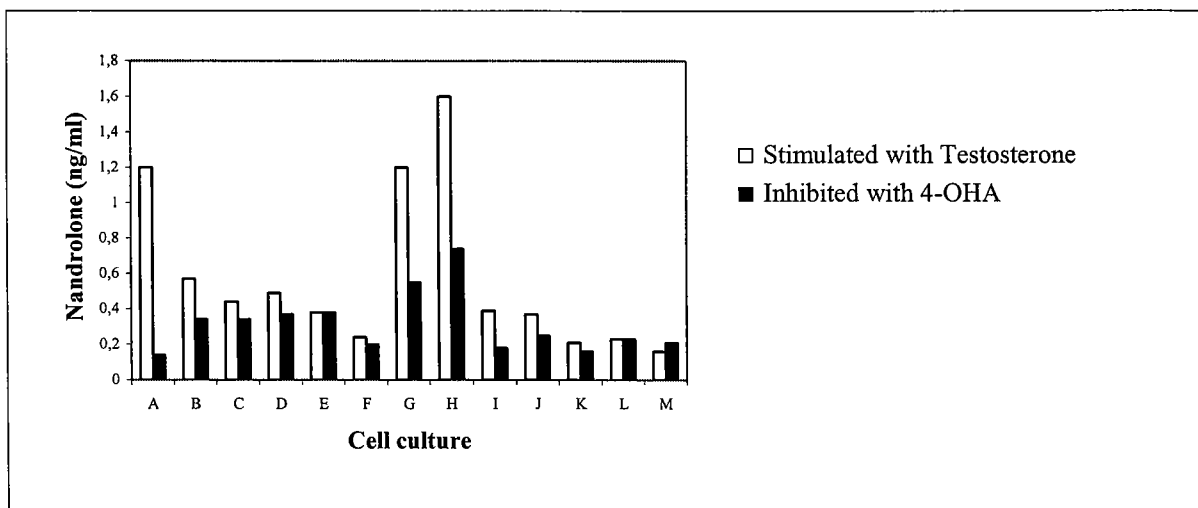
Figure 4. Nandrolone production in granulosa cell cultures.
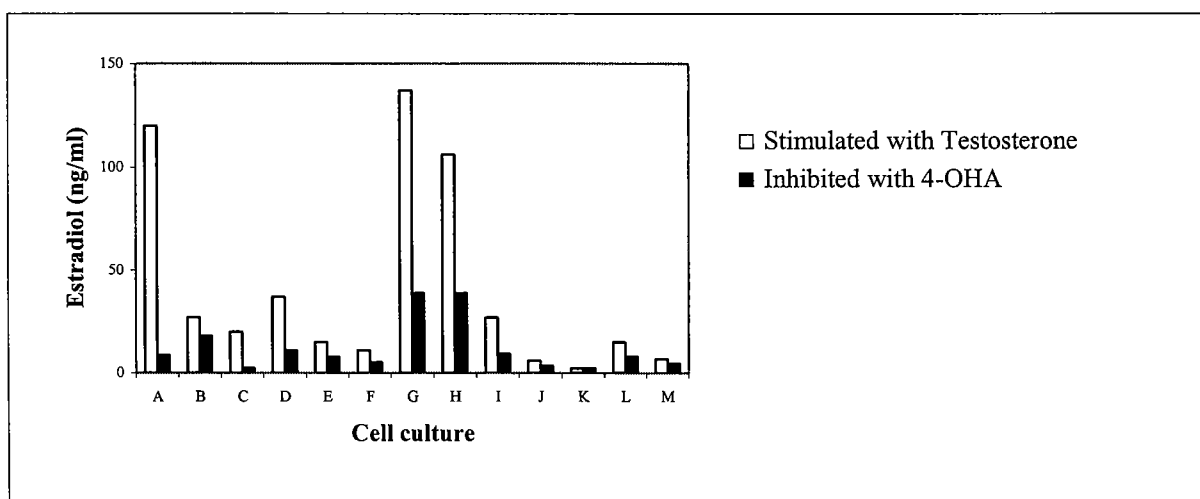Effect of inhibition with aromatase inhibitor 4-OHA.

Figure 5. Estradiol production in granulosa cell cultures.
Effect of inhibition with aromatase inhibitor 4-OHA.

Nandrolone production was inhibited by 4-OHA in 11 of 13 granulosa cell cultures. In 2 cell cultures, no effect could be observed. After inhibition the concentration of nandrolone was very low in some cell cultures, even below the limit of quantitation. The production of estradiol was inhibited between 30 and 90 % by 4-OHA in all cell cultures except from one preparation where the estradiol level was very low. Although a distinct inhibition of nandrolone production can be observed, it is impossible to quantify the degree of inhibition with nandrolone concentrations below the LOQ, it seems like nandrolone production was inhibited in the same manner as estradiol. This implies that the enzyme system aromatase is involved in the production of both estradiol and nandrolone.

In the granulosa cell cultures, where 4-OHA was added without testosterone, no production of nandrolone could be observed. This indicates that 4-OHA neither is a substrate for the aromatase nor interferred with the determination of steroids.

Inhibition with product inhibitor estradiol

The effect of estradiol, added as product inhibitor in the aromatase enzyme system was studied by comparing the steroid production of the granulosa cell cultures, where substrate (2000 nmol/l testosterone) was added with the steroid production from granulosa cell cultures with substrate and the product inhibitor estradiol (1000 nmol/l). To a set of control samples only estradiol (1000 nmol/l) was added. Granulosa cell cultures from 13 patients were studied. The results are presented in Figure 6.
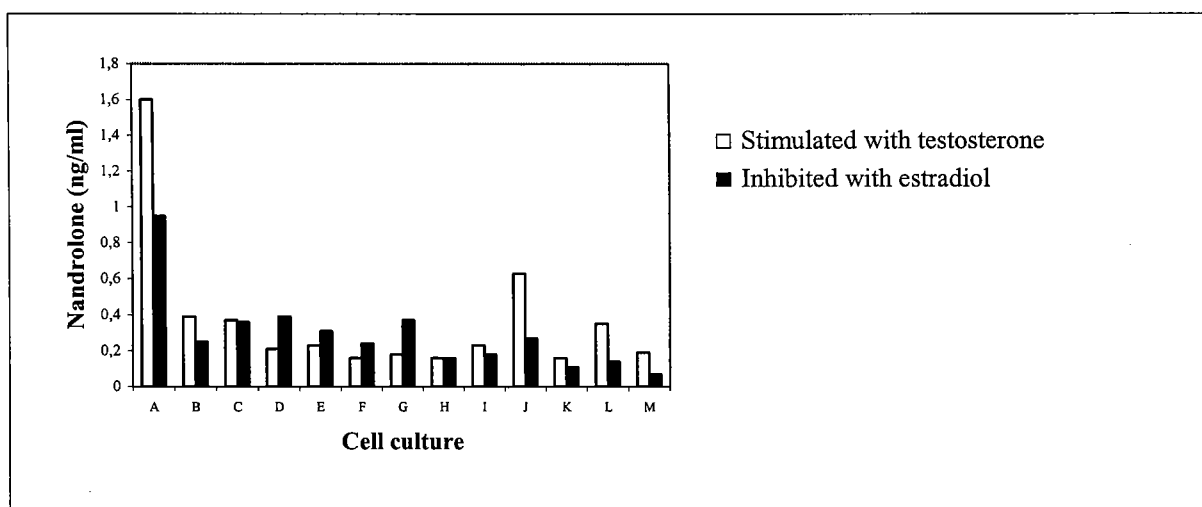

Figure 6. Nandrolone production in granulosa cell cultures.
Effect of inhibition with product inhibitor estradiol.

In 7 of 13 cell cultures nandrolone production was inhibited after incubation with product inhibitor estradiol. In some of the cell cultures the concentration of nandrolone is very low after inhibition, even below the limit of quantitation, thus it is impossible to quantify the magnitude of inhibition. It seems that estradiol is not as efficient as 4-OHA as inhibitor in the enzyme system. This can be due to the affinity for the aromatase that is less strong for estradiol than for a specific inhibitor with androgen structure as 4-OHA.

Conclusion

The production of 19-norandrogens in granulosa-lutein cell cultures was studied with and without testosterone stimulation. In 2 of 24 cell cultures nandrolone-production could be

observed without testosterone stimulation, but the production of 19-norandrogens occurred mainly when the incubation medium had been fortified with testosterone. Nandrolone production was regulated in much the same way as estradiol. Although the production of nandrolone in granulosa-lutein cells was about 1 % of the estradiol, it correlated well with the estradiol production. The production of estradiol and nandrolone was inhibited to the same extent by the aromatase inhibitor 4-hydroxy-androstenedione. Nandrolone production was also inhibited by estradiol when used as product inhibitor. These results indicate that the same enzyme system catalyses the production of estrogens and 19-norandrogens and that nandrolone is produced as a by-product of the aromatisation reaction in granulosa cells of the human ovary.

Acknowledgements

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