Human Reproduction Vol.22, No.12 pp. 3178–3183, 2007 doi:10.1093/humrep/dem328
Advance Access publication on October 20, 2007

Reproductive implication of D-aspartic acid in human pre-ovulatory follicular fluid

G. D'Aniello[1,3], N. Grieco[1], M.A. Di Filippo[1], F. Cappiello[1], E. Topo[2], E. D'Aniello[2] and S. Ronsini[1]

[1]Department of Obstetrics, Gynecology and Physiopathology of Human Reproduction, Hospital S. Luca, 84078 Vallo della Lucania SA, Italy; [2]Department of Biochemistry and Neurobiology, Zoological Stazione A Dohrn, 80121 Napoli, Italy

[3]Correspondence address. E-mail: gdaniello@hotmail.com

BACKGROUND: In the present study, we report that D-aspartic acid (D-Asp) occurs in human ovarian follicular fluid and that a relationship may exist between the concentration of this amino acid and oocyte quality. METHODS: Samples of pre-ovulatory follicle fluid were obtained from 20 patients undergoing an IVF programme. The concentration of D-Asp was measured by using specific high-performance liquid chromatography (HPLC) combined with a D-aspartate oxidase. RESULTS: D-Asp occurs in human follicular fluid at a mean concentration of 14.98 ± 4.51 nmol/ml. A significant difference in the content of this amino acid in the follicular fluid in relation to patient's age exists. In younger women aged 22–34 years (group A), D-Asp was found at a concentration of 19.11 ± 1.91 nmol/ml, whereas in patients aged 35–40 years (group B), it decreased to 10.86 ± 1.22 nmol/ml ($P < 0.01$). In addition, this amino acid was linked to oocyte quality; a relationship exists between D-Asp follicular concentration and the percentage of good quality metaphase II oocytes ($P < 0.01$), as well as the fertilization rate. CONCLUSIONS: In human follicular fluid, D-Asp is present at a relatively higher concentration in younger women than in older patients and there appears to be a relationship between the concentration of D-Asp and fertility outcome parameters. These findings suggest that follicular D-Asp concentration may be considered as an alternative or additional biochemical marker for oocyte quality in patients undergoing IVF programmes.

Keywords: D-aspartic acid; amino acids; human pre-ovulatory follicular fluid; *in vitro* fertilization

Introduction

Since the discovery of D-aspartic acid (D-Asp) in molluscs by D'Aniello and Giuditta (1977), this amino acid has been identified in neuroendocrine tissues of a variety of animal phyla: molluscs, opistobranchs, tunicates, crustaceans, amphibians, reptiles, fishes, birds and mammals including humans (for a review see D'Aniello, 2007). Collecting all the results obtained until now, it appears clear that D-Asp in biological tissues has a double role: (i) it is implicated in the nervous tissue as a possible neurotransmitter or neuromodulator and (ii) it is implicated in the endocrine tissues as a molecule promoter inducing hormonal biosynthesis and release.

Concerning the nervous function, many data suggest that D-Asp promotes the synthesis of proteins involved in the development of the nervous system and also acts as a neurotransmitter/neuromodulator at synapses. For instance, in the brain of bird and mammalian embryos at the last stage of development, D-Asp is present at a very high concentration (300–500 nmol/g), whereas after birth it decreases to low levels (20–40 nmol/g) and remains the same for the rest of life (Dunlop *et al*., 1986; Neidle *et al*., 1990; Hashimoto *et al*., 1993). These events suggest that D-Asp acts as a factor inducing mRNA for the biosynthesis of specific proteins that are necessary for the development of the central nervous system. In adult animals, evidence exists that D-Asp is implicated in neurotransmission. For example: (i) D-Asp is implicated in vision. It is present in the retina of the molluscs *Sepia officinalis* and increases in response to the light intensity (D'Aniello *et al*., 2005b), whereas in the goldfish retina it is capable of potentiating the effects of L-glutamate in the light response (Ishida *et al*., 1981); (ii) a protein able to transport D-Asp from synaptic clefts to the pre-synaptic neurons has been found (Bouvier *et al*., 1992; Kanai *et al*., 1992); (iii) D-Asp is released from rat cerebellum slices by depolarizing stimuli of potassium ions via a calcium channel dependent mechanism (Davies *et al*., 1976; Malthe-Sarenssen *et al*., 1979); (iv) D-Asp occurs in synaptic vesicles (Spinelli *et al*., 2006) and (v) a D-Aspartate oxidase, the enzyme which destroys D-Asp in the neuron (Zaar *et al*., 2002) and in the post-synaptic membrane, exists (D'Aniello *et al*., unpublished data).

Concerning the endocrine function, in 1996 it was demonstrated that injection of D-Asp in rats induces a plasma increase

of LH, testosterone, progesterone and prolactin (D'Aniello *et al*., 1996, 2000a,b). In addition, *in vitro* experiments have demonstrated that D-Asp has a direct action on testes to induce testosterone and progesterone release (D'Aniello *et al*., 2000a). Biochemical approaches suggest that D-Asp is highly concentrated in rat testicular venous blood plasma and testis fluid, in testicular parenchymal cells and in seminiferous tubules (D'Aniello *et al*., 1998), whereas immunohistochemical approaches have demonstrated that D-Asp is localized in the elongated spermatids, the most mature germ cells (Sakai *et al*., 1998). Further *in vitro* experiments, consisting of the incubation of isolated hypothalamus with D-Asp, have indicated that D-Asp elicits the release of mammalian GnRH (D'Aniello *et al*., 2000a), the peptide hormone responsible for LH and FSH release from the pituitary cells. In addition, D-Asp constitutes the endogenous precursor for *N*-methyl-D-aspartic acid (NMDA) biosynthesis (D'Aniello *et al*., 2000a), and previous studies have demonstrated that NMDA is the specific molecule that enhances the release of the GnRH from the hypothalamus (Price *et al*., 1978; Gay *et al*., 1987; Ondo *et al*., 1988; Pohl *et al*., 1989; Downing *et al*., 1996). Recent studies have demonstrated that human seminal fluid and spermatozoa contain D-Asp and that a direct relationship exists between D-Asp concentration and semen quality, meaning that D-Asp is involved in male reproduction (D'Aniello *et al*., 2005a). In this study, we show that D-Asp is also present in human pre-ovulatory follicular fluid and that a link may exist between the concentration of D-Asp and the age of the patients as well as oocyte quality.

Materials and Methods

Patients

Follicular fluid specimens were obtained from 20 Caucasian patients, aged 31.9 $\pm$ 5.83 (range 22–40 years), referring to the Department of Obstetrics, Gynaecology and Human Reproduction, Hospital S. Luca, Vallo della Lucania, Salerno, Italy, in the period between June 2006 and December 2006 for an IVF program. All couples included in the study presented a severe male factor for infertility and were candidates for an ICSI cycle. Sperm concentration and motility were evaluated with the use of a fixed-depth counting chamber Makler Chamber (Sefi Medical Instruments, Haifa, Israel) and analysed according to the World Health Organization guidelines (WHO, 1999). Sperm morphology was evaluated by staining with May–Grunwald–Giemsa according to Kruger's criteria (Menkveld *et al*., 1991) that consider the percentage of abnormal heads, abnormal tails, midpiece abnormalities and immature forms, with a minimal value of normal forms $\geq$14%. A severe male factor was defined by a sperm concentration $<10\times^{6}$/ml, a progressive motility $<$5% and a normal sperm morphology under 8%.

Informed consent was obtained from each patient before oocyte collection, and the research protocol was approved by the Institutional Review Board (IRB) of this hospital.

Ovarian stimulation and oocyte retrieval

Ovarian stimulation was achieved using a conventional short protocol in which s.c. 0.1 mg injections of GnRH agonist triptorelin acetate (Decapeptyl, Ipsen SPA, Italy) starting on Day 1 were followed by gonadotrophin, rFSH (Gonal F®, Serono Pharma, Italy), administered s.c. in individual doses for each patient starting on Day 3 of the cycle. Follicular growth was monitored by ovarian ultrasound and serum 17β-estradiol (E_2) measurement from Day 5 on alternate days.

The induction of ovulation with urinary hCG (Gonasi®, AMSA, Rome, Italy) was performed when the leading follicle reached 18–20 mm in diameter and when the serum E_2 concentration per follicle was 150–200 ng/l.

Follicular fluid collection, oocyte collection, insemination and embryo culture

Pre-ovulatory ovarian follicular fluid was collected during transvaginal ultrasound guided oocyte retrieval. Only follicular fluid samples which were macroscopically free from blood were retained for further determinations as described by Józwik and Wolczynski (1998). Sampling was done when follicles ranged from 18 to 22 mm in diameter. The follicular fluid samples were collected into capped disposable polypropylene tubes and mixed with an equal volume of 0.5 M trichloroacetic acid (TCA) and centrifuged at 15 000*g* for 30 min. The supernatant was purified on cation-exchange resin and used for the HPLC determination of D-Asp as described below.

The oocytes'–cumulus complexes were transferred to flushing medium (Global Life, USA) and then transferred individually in 50–100 μl drops of human tubal fluid (HTF) (Global Life) supplemented with human serum albumin (Global Life) under mineral oil (Global Life), in Nunc dishes (Nalge Nunclon International, Rochester, NY) incubated at 37°C in an atmosphere of 5% CO_2 in air. After denudation obtained with hyaluronidase (Global Life), oocytes were observed and evaluated with use of the $\times$40 objective plus the 1.5 magnifying lens of an Olympus inverted microscope (Olympus America, Melville, NY), equipped with Hoffman Modulation Optics. Mature oocytes (MII stage) were divided into two groups, according to their quality:

Group 1: good quality metaphase II oocytes with a clear, moderately granular cytoplasm, a small perivitelline space, a clear zona pellucida and an intact polar body according to Veeck (1988).

Group 2: poor quality metaphase II oocytes with morphological variations and changes in colour, granularity and homogeneity of the cytoplasm, variations of perivitelline space and zona pellucida (Veeck, 1988; Van Blerkom, 1990).

Sperm preparation and insemination was performed in HTF medium; insemination was performed by ICSI, and embryo culture was in GBM medium (Global Life) supplemented with human serum albumin (Global Life) under mineral oil (Global Life). A fertilization check was performed at 16 h post-insemination on an inverted microscope.

The fertilization rate (FR) was defined as the number of two pronuclei cells over the number of oocytes inseminated.

Purification and determination of D-Asp

Follicular fluids (3–4 ml) were mixed with an equal volume of 0.5 M TCA and centrifuged at 15 000g for 30 min and then purified on a column (1 $\times$ 4 cm) of cation-exchange resin (AG 50W-X8; 200–400 mesh, Bio-Rad, Hercules, CA, USA), and after absorption, the column was washed with 12 ml of 0.01 M HCl followed by 3 ml distilled water. After that, 8 ml of 4 M NH_4OH was passed through the column and the eluent, containing purified amino acids, including D-Asp, was collected and dried by evaporation in small Petri dishes under a hood over a warm plate at 40–50°C. The residue was dissolved 2 ml distilled water and used for the HPLC determination of D-Asp according the described method (D'Aniello *et al*., 2005a, b) modified as follows: 20 μl of the purified sample was mixed with 50 μl of 0.1 M Tris–HCl, pH 8.2, 100 μl of 0.1 M trisodium phosphate buffer, pH 10.0 and 20 μl of OPA-NAC reagent (20 mg

o-phthaldialdehyde plus 10 mg of *N*-acetyl-L-cysteine in 2.0 ml of methanol). After 2 min, distilled water was added to the mixture to a final volume of 1.0 ml, mixed, and 100 μl was injected on a Supelcosil C-18 HPLC column (0.45 × 25 cm, 5 μm beads; Supelco, Bellefonte, PA, USA). Amino acids were eluted using buffer A, consisting of 5% acetonitrile in 30 mM citrate phosphate buffer, pH 5.6, and buffer B consisting of 90% acetonitrile in double distilled water. The gradient was the following: 0–15% B over 10 min, 15–100% B over 5 min, staying at 100% B for 5 min and returning to 100% A in 1 min. The flow rate was 1.2 ml/min. Amino acids were detected fluorometrically at an excitation wavelength of 325 nm and an emission wavelength of 415 nm. D-Asp elutes with a peak at 5.3 min, followed by L-Asp 0.7–0.8 min later and then other amino acids (Fig. 1). In order to confirm that the peak eluting at 5.3 min was actually D-Asp, 20 μl of the purified sample were mixed with 50 μl of 0.1 M Tris–HCl, pH 8.2, and 2.0 μl of purified D-aspartate oxidase obtained by over expression (D'Aniello *et al.*, 2005b) at a concentration of 2.0 mg/ml and incubated at 37°C for 30 min. Then this oxidized sample was treated as above with phosphate buffer and OPA-NAC, and 100 μl of this sample was used for HPLC analysis. The almost complete reduction of the peak at 5.3 min confirmed the presence of D-Asp in the earlier samples (Fig. 2). In order to know the exact concentration of D-Asp and L-Asp, the following standard mixture of

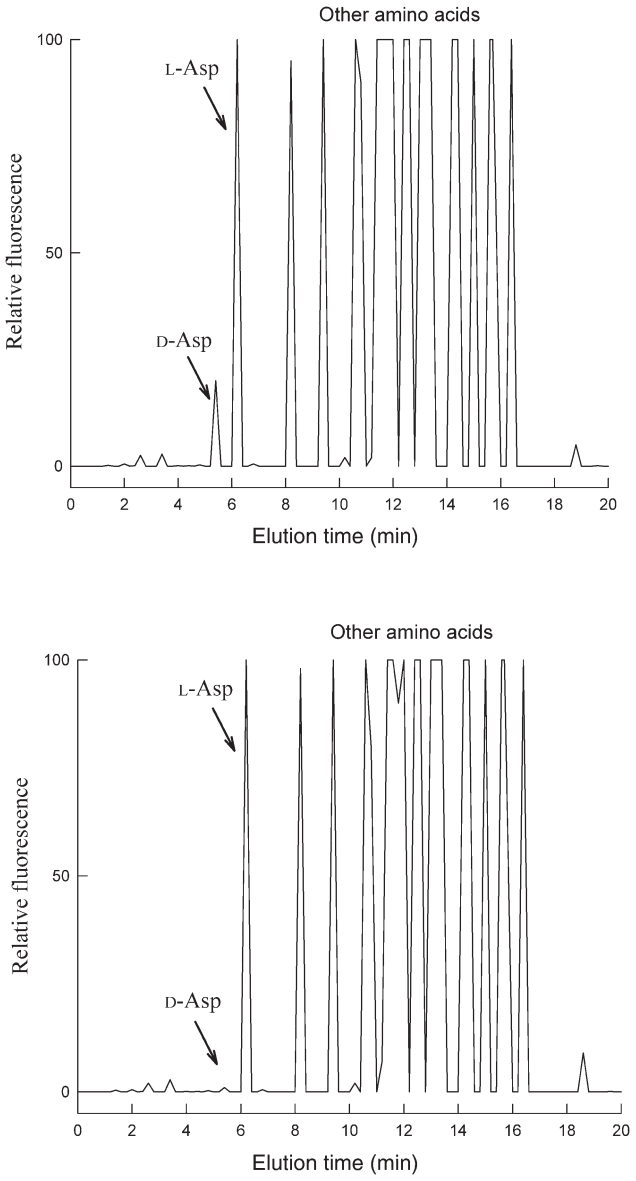

Figure 1: HPLC chromatography of D-Asp and L-amino acid standards

Typical example of HPLC analysis of a standard amino acid mixture. Upper panel shows an HPLC chromatography profile of D-Asp and other L-amino acid standards. The areas of the peaks correspond to an amount of 10 pmoles of D-Asp and an amount of 50 pmoles for each L-amino acid. The arrows indicate the elution time of free D-Asp and L-Asp. Lower panel shows the HPLC analysis of the same sample, but after incubation with D-Aspartate oxidase, the enzyme which specifically oxidizes D-Asp. The reduction of the peak corresponding to D-Asp confirms the presence of D-Asp

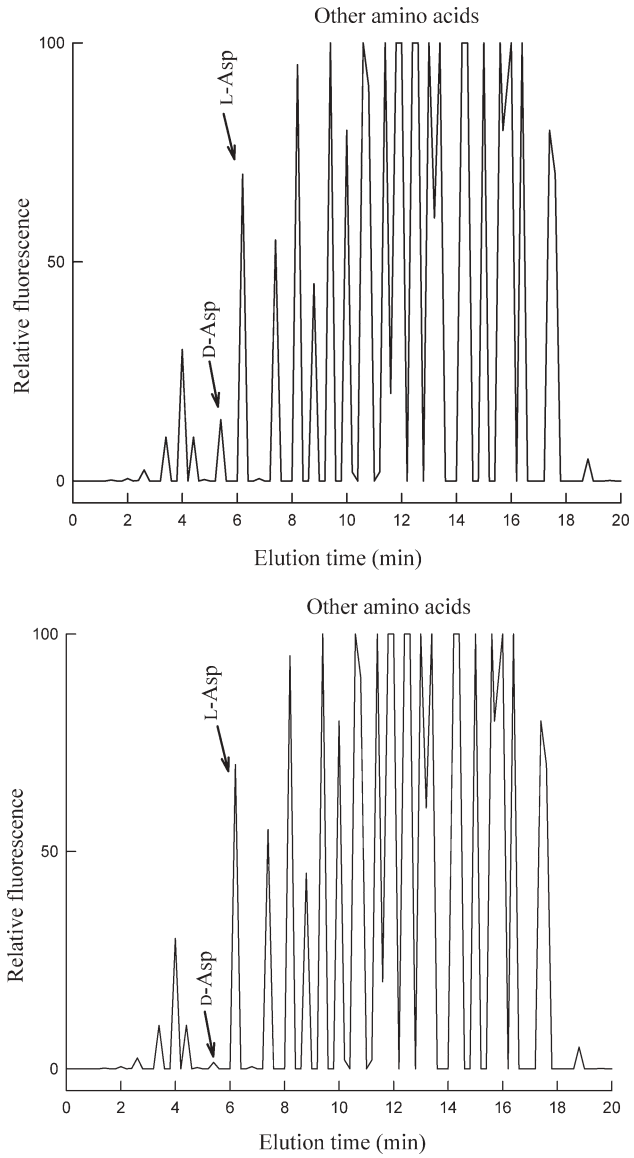

Figure 2: Determination of D-Asp in a follicular fluid sample by HPLC

Typical example of HPLC analysis of a follicular fluid sample. The upper panel shows the HPLC analysis of D-Asp content in 2 μl of the original follicular fluid. The arrows indicate the elution time of free D-Asp and L-Asp. The lower panel shows the HPLC analysis of the same sample, but after treatment with D-aspartate oxidase. Again in this case, the reduction of the peak corresponding to D-aspartate oxidase confirms the presence of D-Asp

amino acids was prepared: 100 μl of a mixture of a 17 different L-amino acids (Sigma Chemical, St. Louis, MO, USA), each at a concentration of 0.01 μmol/ml, 20 μl of D-Asp at a concentration of 0.01 μmol/ml, 50 μl of 0.1 M Tris–HCl, pH 8.2, 100 μl trisodium phosphate buffer, pH 10.0 and 10 μl of OPA-NAC. After 2 min double distilled water was added to a final volume of 1 ml, and 100 μl was injected on the column and the HPLC analysis was continued as above. In order to calculate the concentration of D-Asp, the area of the peak of D-Asp of the sample (area of D-Asp of the sample minus the area of D-Asp obtained after treatment with D-aspartate oxidase) was compared with the area of D-Asp obtained from the standard curve.

Statistical analysis

Results were expressed as means ± SD. Data were analysed with the SPSS version 12.0 (SPSS Inc. USA). The statistical analysis was performed using Student's *t*-test (two-tailed values). Fisher's exact test was used to assess differences between groups. The Mann–Whitney *U*-test was applied to test differences between groups for continuous variables with non-parametric distributions. A *P*-value <0.05 was considered statistically significant.

Results

Using the described method for the purification of D-Asp from follicular fluid on cation-exchange resin and the method for the determination of D-Asp by the HPLC associated with the use of D-aspartate oxidase, we found a significant amount of D-Asp in pre-ovulatory follicular fluid of all patients examined (Table I). The mean concentration of D-Asp determined on 20 samples of follicular fluid obtained from women aged between 22 and 40 years was found to be 14.98 ± 4.51 nmol/ml of follicular fluid (Table I). In order to know if there was a difference in D-Asp content in relation to patient's age, samples were divided into two groups: group A, which consisted of 10 patients aged 22–34 years (26 ± 3.68yrs), and group B formed by 10 patients aged 35–40 years (37 ± 1.49). The results obtained from this investigation indicated that a significant difference in D-Asp concentration in follicular fluid of patients with different age exists. Specifically, in younger patients (group A), a follicular fluid D-Asp concentration of 19.11 ± 1.91 nmol/ml was found, whereas in older patients (group B), this concentration was about half that of group A (10.86 ± 1.22 nmol/ml). The difference was statistically significant for a *P*-value <0.01.

In addition to the biochemical determination of D-Asp in follicular fluid, we also detected for each patient other physiological parameters concerning the number of oocytes collected, the number of MII oocytes (Table II), the number of good and poor quality metaphase II oocytes and, finally, the FR (Table III).

Interestingly, we found that a direct relationship may exist between the concentration of D-Asp in the follicular fluid and the number of oocytes collected as well as the oocyte quality. In fact, as is shown in Table II, in the group of patients aged between 22 and 34 years, the mean number of oocytes collected for each patient was 14 ± 2.9, while the mean number of oocytes collected in the patients aged between 35 and 40 years was 7.1 ± 1.4 ($P < 0.01$). However, almost all of these oocytes collected were at MII maturation status in both groups (group A: 85.0%; group B 95.8%, $P > 0.05$).

Table I. Content of D-Asp in follicular fluid in 20 patients aged 22–40 years.

	Age (years)	D-Aspartic content (nmol/ml follicular fluid)
Group A		
	22	18.2
	23	18.0
	24	19.2
	24	22.9
	26	21.5
	27	19.3
	28	18.5
	29	19.7
	30	17.4
	34	16.4
Mean ± SD	26.7 ± 3.68	19.11 ± 1.91*
Group B		
	35	13.0
	36	12.1
	36	11.2
	36	10.7
	36	10.5
	37	11.2
	38	10.4
	38	11.2
	38	9.8
	40	8.5
Mean ± SD	37 ± 1.49	10.86 ± 1.22*
Mean ± SD of both groups	31.85 ± 5.94	14.98 ± 4.51

*Value significant for a $P < 0.01$.

Furthermore, a statistically significant direct relationship exists between D-Asp concentration in follicular fluid and the number of MII oocytes of good quality and the FR among groups. In fact, in women of group A, D-Asp follicular fluid concentration was 19.11 ± 1.91 nmol/ml and the number of oocytes of good quality was 11.5 ± 1.8 (Table III), which corresponds to 82% of total oocytes collected (14.0 ± 2.9; Table II); instead, in women of group B, D-Asp follicular fluid concentration was 10.8 ± 1.22 nmol/ml and the number of good quality oocytes was 2.4 ± 0.4 (Table III), which corresponds to 33% of total oocytes collected (7.1 ± 1.4; Table II). These values are statistically significant at $P < 0.01$.

In addition, to evaluate the IVF outcome, two MII oocytes of good quality from each patient were used for a standard procedure of sperm injection (ICSI). The results indicated that the FR of group A was increased compared with group B. In fact, in group A the FR was of 96.7%, whereas in group B it was 87.0% ($P < 0.05$, Table III).

Discussion

In this study, we report that in pre-ovulatory human ovarian follicular fluid there is present a considerable quantity D-Asp in free form and that its concentration could be related to the patient's age and to the quality of oocytes. Previously, we found that D-Asp is present in male reproductive tissues, such as seminal fluid and spermatozoa, and found a direct relationship between semen quality and D-Asp concentration (D'Aniello *et al.*, 2005a). Thus, those data and the results

Table II. Composition of D-Asp follicular concentration and number of oocytes collected and number of MII oocytes among groups.

	Mean of D-Asp in follicular fluid (nmol/ml)	Mean of oocytes retrieved	Mean of MII oocytes
Patients of the group A ($n = 10$, age 22–34)	19.1 ± 1.91*	14.0 ± 2.9*	11.9 ± 1.7 (85.0%)[a]
Patients of the group B ($n = 10$, age 35–40)	10.8 ± 1.22	7.1 ± 1.4	6.8 ± 1.3 (95.8%)

Values are means ± SD obtained from 10 patients of each group. [a]is the percentage of MII oocytes on total retived. *Value significant for a $P < 0.01$.

found in the present investigation indicate that D-Asp could have a role in male and female reproduction. This hypothesis is in agreement with results previously found in rats. In fact, it was found that testes from sexual mature rats contain D-Asp at higher concentrations, whereas in younger rats this amino acid was at significantly lower values (D'Aniello *et al*., 1996, 1998, 2000a; Sakai *et al*., 1998). In addition, we also demonstrated that in this animal, D-Asp is concentrated in the endocrine glands and furthermore, exogenous administration of D-Asp induces an increase in LH and testosterone in the serum.

Previous studies, have reported the amino acid composition of human follicular fluid obtained during surgery from women with polycystic ovaries (Edwards *et al*., 1974; Velázquez *et al*., 1977; McNatty *et al*., 1981) or the amino acid content of follicular fluid during a natural or stimulated cycle (Menezo *et al*., 1982; Jozwik *et al*., 2006). Edwards *et al*. (1974) first reported the source, properties, composition and function of human follicular fluid in his review, suggesting that free amino acids participate actively in various physiological processes. Velazquez *et al*. (1977) studied the amino acid and protein composition in human follicular fluid obtained during surgery by puncture of follicles (~0.5 cm diameter) in six cases of polycystic ovaries. They found that amino acid concentrations were higher in follicular fluid than in blood plasma (except for cystein) indicating that follicular fluid could be formed by filtration of the blood plasma. In addition, other data about the amino acids and amine compounds in preovulatory ovarian follicular fluid have been reported by Velazquez *et al*. (1977) and Jozwik *et al*. (1999c, 2001) showing that glutamine, urea, taurine and threonine were the amino acids found at a comparative greater concentration and that their function was attributed to maintainance of an osmotic balance between the extracellular fluid and the follicular cells.

Concerning D-Asp, no previous studies on the concentration of D-amino acids in follicular fluid of IVF patients have been carried out. L-Asp concentration in the follicular fluid is not particularly high compared with glutamine and urea (Velazquez *et al*., 1977; Jozwik *et al*., 2006), however, it is still found at a substantial concentration, leading us to hypothesize that the D-Asp found by us in follicular fluid may originate from L-Asp by the action of a racemase. This phenomenon, in fact has been demonstrated to occur in rat brain and testis (Shell *et al*., 1997; Wang *et al*., 2000a; Wolosker *et al*., 2000).

Follicular fluid is clearly involved in the migration of the mature oocyte to the oviduct (Blandau *et al*., 1969) and its role in *in vitro* experiments for the capacitation of spermatozoa has been defined repeatedly (Yanagimachi *et al*., 1969; Hicks and Pedron, 1972). The effect of amino acids present in human follicular fluid on murine embryo development has been studied by Nakazawa *et al*. (1997), suggesting that the concentrations of amino acids found in follicular fluid are more effective and safer for embryo culture than those in other media currently in use.

Moreover, a previous study has demonstrated that D-Asp is able to induce the synthesis and release of LH in rat (D'Aniello *et al*., 1996). In fact, if a rat receives via i.p. sodium D-aspartate (1–2 μmol/g of body weight), after 2–5 h, the levels of LH increase about three times (from a basal value of 3.7 ng/ml of serum to 9.1 ng/ml). Furthermore, more recent studies, carried out on human subjects, have demonstrated that after orally consumption of 10 ml of sodium D-aspartate solution at a concentration 2 M (total 2.6 g D-aspartate) (Dadavit®-Pharmaguida, Italy) for a time period of 10–15 days, serum LH levels are increased by about 1.5 times compared with the control for almost the 85% of subjects cases (unpublished data).

As known, the mid-cycle LH surge is involved in the maturation of the oocyte and follicular luteinization. Recent studies indicate that aberrations in the follicular fluid LH concentration may be detrimental to oocyte fertilization and subsequent embryo development (Stranger *et al*., 1985; Lewinthal *et al*., 1986; Chappel and Howles, 1991; Cohlen *et al*., 1993). In addition, Verpoest *et al*. (2000) have reported IVF of oocytes

Table III. Comparison of D-Asp follicular concentration and oocytes quality among groups.

	D-Asp in follicular fluid (nmol/ml)	No. oocytes MII good quality	No. oocytes MII bad quality	FR (%)
Patients of the group A $n = 10$, age 22–34	19.1 ± 1.91*	11.5 ± 1.8*	0.4 ± 0.09*	96.7**
Patients of the group B $n = 10$, age 35–40	10.8 ± 1.22	2.4 ± 0.4	4.4 ± 1.2	87.0

Values are means ± SD; FR indicates the fertilization rate calculated only on two MII oocytes for each patients. *Value significant for a $P < 0.01$; **Value significant for a $P < 0.05$.

from patients with decreased concentrations of follicular fluid LH produces lower FRs, confirming that diminished follicular LH concentrations may cause decreased oocyte quality. Since it has also been demonstrated that LH increases ovarian sensitivity to FSH and that an LH deficit may affect the competence of oocytes to develop into embryos able to implant (Filicori, 1999), our findings concerning the occurrence of D-Asp in follicular fluid, and its probable relationship to oocyte quality, as well as the implication of D-Asp in LH increase in rat (D'Aniello *et al*., 1996, 2000a), support the hypothesis that the human D-Asp may have a role in improving oocyte quality.

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