Institute of Experimental Morphology and Anthropology with Museum

Acta morphologica et anthropologica, 11
Sofia • 2006

Importance of Androgens, Estrogens and Gonadotrophins for Rat Leydig Cell Development and Function through Puberty to Adulthood

N. Atanassova, C. McKinnell, M. Walker, R. M. Sharpe*

**Institute of Experimental Morphology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia*
MRC, Human Reproductive Sciences Unit, Edinburgh, UK

The aim of the present study was to investigate the ontogeny of Leydig cell development and function through puberty to adulthood in rats in which hormonal environment (gonadotrophins, androgens and estrogens) in neonatal life had been manipulated experimentally. Quantification of LC development (3β-HSD positive cells) and function (plasma T levels) demonstrated that treatment with GnRHa and DES impaired testis growth, total LC volume per testis and T production during puberty compared to controls. The final LC number per testis in adulthood was comparable with control values. T-levels were significantly reduced in DES but not in GnRHa treated rats. Our results showed that manipulation of hormone environment during the first 2-3 weeks of postnatal life did not produce any major consistent effect on final LC number in adulthood. It can be suggested that the factors determining ALC number are already predetermined at birth or are not dependent/sensitive to gonadotrophins, androgens and estrogens in the neonatal/ pubertal period.

Key words: Leydig cells, androgens, estrogens, gonadotrophins, rat testis.

Introduction

Leydig cells (LC) as a main place for androgen production play a central role in development and function of the testis and male reproductive tract. The adult population of LC develops during puberty from presumptive precursor cells through three distinct phases – progenitor (PLC), immature (ILC) and adult (ALC) LC [4]. The mechanisms that regulate differentiation of precursor into adult LC and their numbers are incompletely understood, although it is known that hormones such as LH, FSH, thyroid hormones, androgens and estrogens and growth factors (IGF-I, TGF-α and β, PDGF) all play a role in LC development [1, 9].

Estrogens are reported to play negative role in regulating of rat LC and they inhibit both steroidogenesis and differentiation of LC, acting as negative feedback regulators [1, 8]. Data derived from studies on LC regeneration after treatment with EDS demonstrated that proliferation of PLC was unaffected by estrogens. In contrast, differentiation of these PLC into ILC appeared to be inhibited by estrogens, whereas androgens

play a positive role in this process [6]. It is becoming evident that above-mentioned hormones play a role in regulation of LC development but exactly which hormones/ other factors regulate final LC number in the adult testis has not been clearly defined. Studies on hypothyroid rat indicated that the number of Sertoli cells per testis determined the number of LC [7].

Our previous studies revealed that hormone manipulations in neonatal rats that dramatically changed gonadotrophin, androgen and estrogen levels affected Sertoli cell maturation and adult Sertoli cell number [2]. In this respect the aim of the present study was to investigate the ontogeny of Leydig cell development and function through puberty to adulthood in rats in which hormonal environment in neonatal life had been manipulated experimentally.

Materials and Methods

Animals and treatments: The experimental manipulation of hormonal balance in male neonatal rats was performed as follow: beginning on postnatal day 2, rats were subjected to one of the following treatments administered by s.c. injection: a) DES at a dose of 10,1 or 0.1 μg in 20 μl corn oil on days 2, 4, 6, 8, 10 and 12; b) 10mg/kg of long acting GnRH-antagonist (GnRHa, Antarelix) in 20 μl 5% mannitol on days 2 and 6; c) 10 μg DES as in (a) + GnRHa as in (b); d) 0.1 μg DES as in (a) + GnRHa as in (b); e) 10μg DES as in (a) + 200 μg testosterone at the same regimen; f) 20 μl corn oil (vehicle) as control. Rats from all treatment groups were subsequently sampled on day 18, 25, 35, 75; testes were weighed and fixed in Bouni's and embedded in paraffin.

Immunohistochemistry for 3β-HSD: Leydig cells were immunostained according to the standard ABC-HRP method [10] using rabbit polyclonal antibody against 3β-HSD, kindly provided as a gift form Prof. I. Mason (Edinburgh University).

Quantification of Leydig (3β-HSD positive) cell volume and number per testis: The volume of 3β-HSD positive cells per testis was determined using point-countung methods similar to those outlined previously [2]. The differences were that points falling over 3β-HSD positive cytoplasm or over the nuclei of cells with 3β-HSD positive cytoplasm were scored and converted to absolute volumes per testis and then summed (cytoplasm + nuclear) to get the total LC volume. LC number (millions) per testis was calculated as described previously [10]

Measurement of plasma testosterone levels: T- levels were measured using an enzyme-linked immunosorbent assay [2].

Statistics: Comparison of the different parameters for the various treatment groups was made using Student's t-test.

Results

Cells immunoreactive for 3β-HSD were classified as Leydig cells [2] and they were seen at all ages in all treatment groups and were located in testicular interstitium between seminiferous tubules. The number of 3β-HSD immunopositive cells varied according to age and treatments. There was no difference between the various treatment groups in the intensity of 3β-HSD immunoexpression in LC cytoplasm (Fig.1).

As LC cytoplasm volume is related to their functional activity, measurements of LC nuclear volume may not give the full picture. That's why we used in the present study the total volume (nuclear + cytoplasm) per testis which produced a pattern of results similar to those shown for nuclear volume.

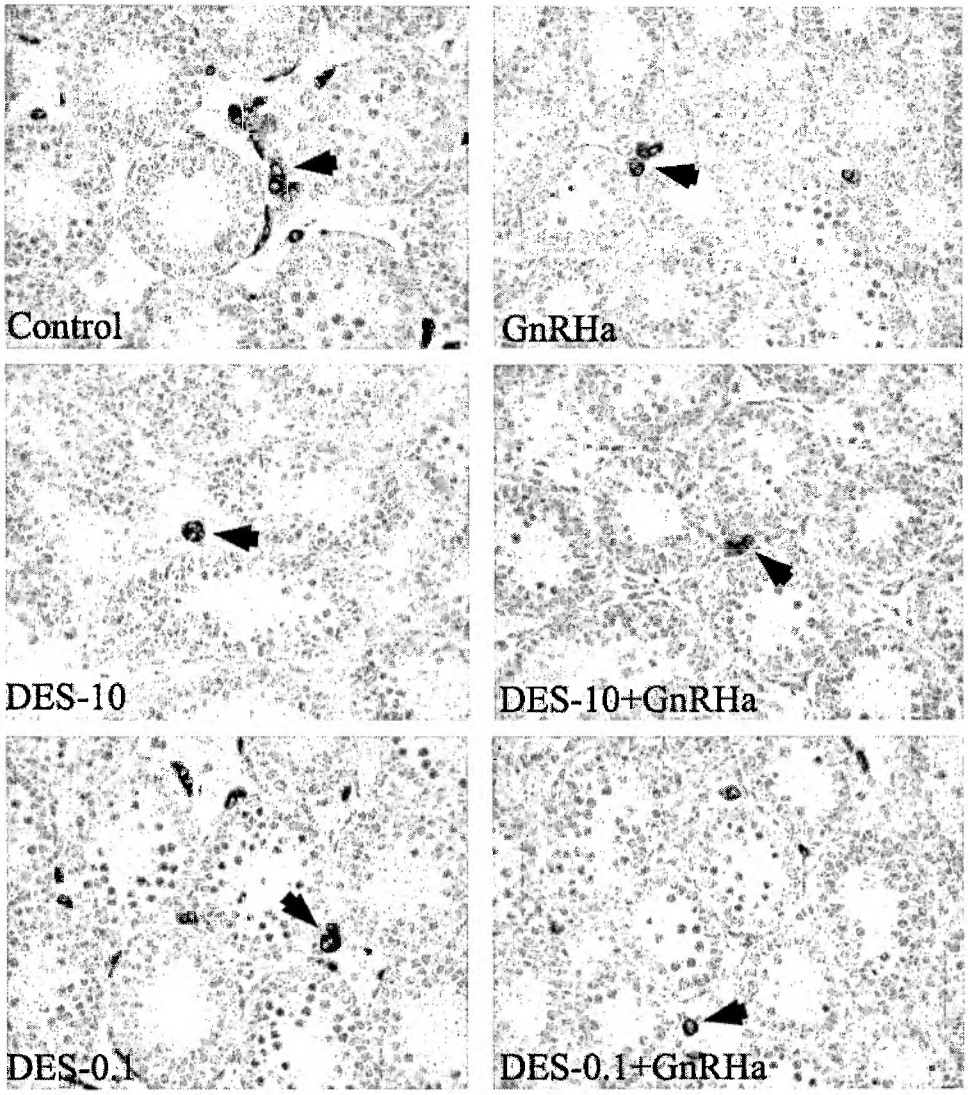

Fig. 1. General morphology of the testis and immunoexpression of the Leydig cell marker enzyme 3β-HSD (arrows) on day 18 in controls and rats treated neonatally with GnRHa or DES alone or combined treatment with GnRHa+DES. Note a strong reaction in LC cytoplasm. × 400

Quantification of LC development (3β-HSD positive cells) and function (plasma T levels) demonstrated that treatment with GnRHa impaired testis growth, total LC volume per testis and T production during puberty compared to controls (Fig. 2, 3, 4). The final LC number per testis in adulthood was slightly lower but comparable with control values (Table 1). Testosterone levels in adult GnRHa treated rats were within the lower end of normal range. Treatment with different doses of DES (10-, 1-, 0.1μg) caused dose-dependent reduction in testis growth, total LC volume and T levels during development (Fig. 2, 3, 4). In adult DES animals LC number was comparable with controls and slightly lower in dose dependent manner (Table 1). Testosterone levels remained

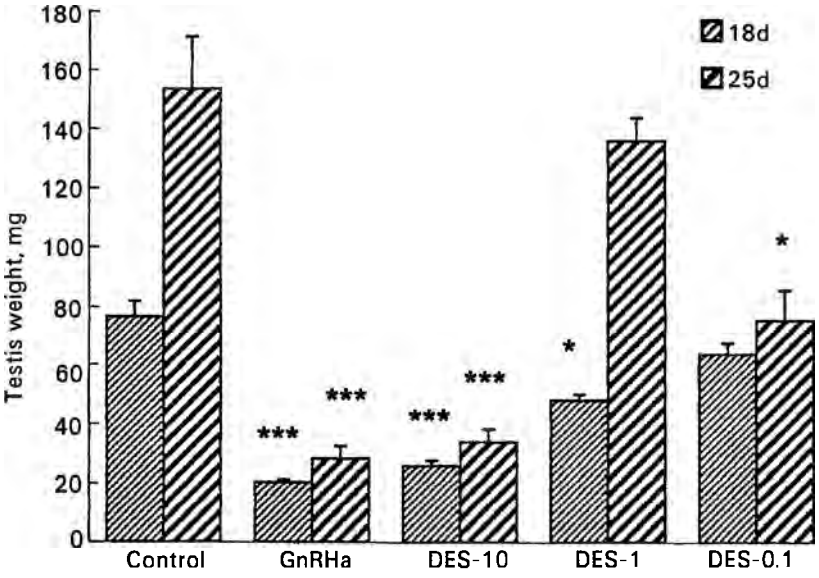

Fig. 2. Testis weight on day 18 and 25 in control and rats treated neonatally with GnRHa or different doses of DES. Data represent mean ± SE (n=5 per group; * p<0.05, ** p< 0.01, *** p< 0.001 in comparison with control value)

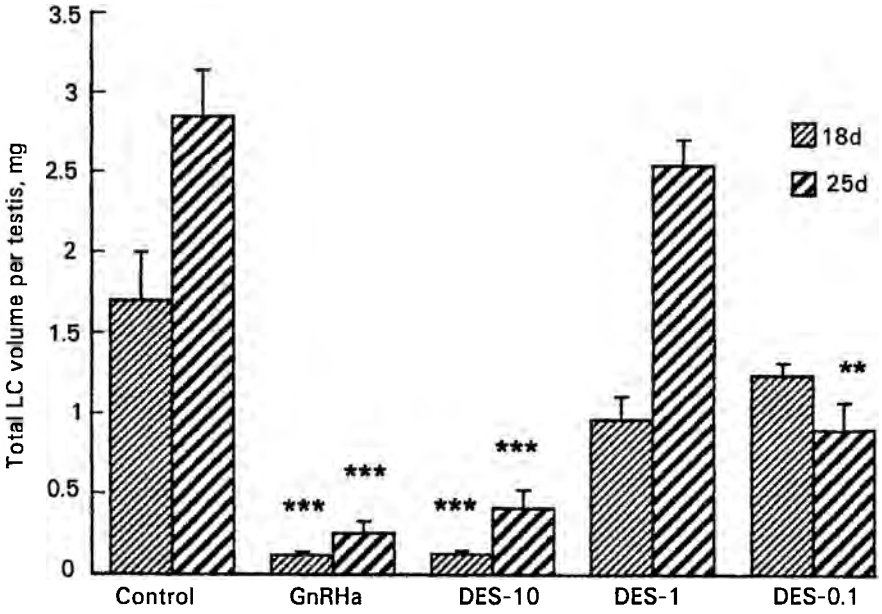

Fig. 3. Total Leydig cell volume (nuclear + cytoplasmic) per testis on day 18 and 25 in control and rats treated neonatally with GnRHa or different doses of DES. Data represent mean ± SE (n=5 per group; * p<0.05, ** p< 0.01, *** p< 0.001 in comparison with control value)

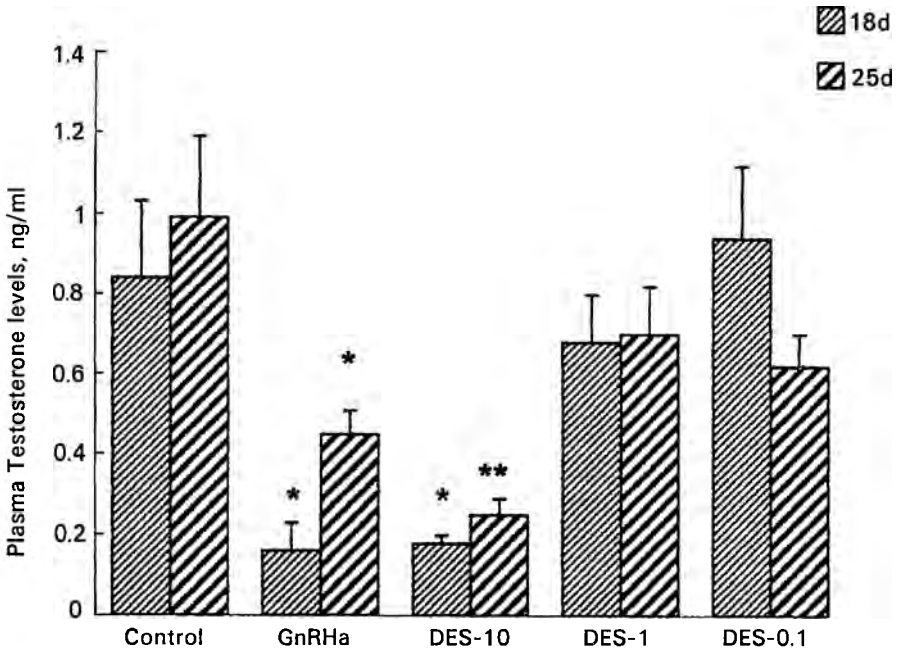

Fig. 4. Plasma testosterone levels on day 18 and 25 in control and rats treated neonatally with GnRHa or different doses of DES. Data represent mean ± SE (n=5 per group; * p<0.05, ** p< 0.01, *** p< 0.001 in comparison with control value)

Table 1. LC number (millions) per testis, testosterone levels and testis weight in adulthood in controls and in rats treated neonatally with GnRHa or different doses of DES. Data represent mean ± SE (n = 3-5 per group; * p<0.05, ** p< 0.01, *** p< 0.001, NS – non significant, in comparison with control value)

Treatment	LC number × 10^6 per testis	T levels (ng/ml)	Testis weight (mg)
Control	32.3 ± 6.6	2.18 ± 0.41	1913 ± 26
GnRHa	26.0 ± 3.6 NS	1.58 ±0.29 NS	1088 ± 60***
DES-10 µg	23.1 ± 3.7 NS	0.36 ±0.09***	966 ± 31***
DES-1 µg	27.0 ± 6.0 NS	0.63 ± 0.13***	1454 ± 141*
DES-0.1 µg	32.3 ± 5.4 NS	1.35 ± 0.22*	1737 ± 101 NS

Table 2. Total LC absolute volume per testis, testosterone levels and testis weight on day 18 in controls and in rats treated neonatally with GnRHa or/and DES. Data represent mean ± SE (n=5 per group; * p<0.05, ** p< 0.01, *** p< 0.001; NS – non significant, in comparison with control value)

Treatment	Total LC volume	T levels (ng/ml)	Testis weight (mg)
Control	1.70 ± 0.30	0.84 ± 0.19	76.63 ± 5.43
GnRHa	0.12 ± 0.02 ***	0.16 ± 0.07 *	20.38 ± 0.67 ***
DES-10 µg	0.13 ± 0.02 ***	0.18 ± 0.02 *	25.88 ± 1.64 ***
DES-10 µg+GnRHa	0.10 ± 0.02 ***	0.176 ± 0.02 *	23.10± 0.34 ***
DES-0.1 µg	1.24 ± 0.08 NS	0.94 ± 0.18 NS	64.0 ± 3.83 NS
DES-0.1 µg+ GnRHa	0.30 ± 0.02 **	0.18 ±0.04 *	26.10 ± 0.72 ***
DES-10 µg+TE	0.08 ± 0.03 ***	2.42 ± 0.39 **	46.73 ± 1.70 **

significantly subnormal and dose-dependently changed. Dose-dependent effect of DES on LC volume was seen on day 35, as well but it tended to recover in GnRHa but not in DES-10 μg treated rats, even significant suppression of T levels (data not shown).

Combined treatment with DES-10 μg + GnRHa on day 18 did not produce greater adverse effect on investigated parameters than either treatment alone (Table 2). Combined treatment with DES-0.1 μg + GnRHa greatly reduced LC volume compared to controls and DES-0.1 μg alone but it was less decreased than that induced with GnRHa.When testosterone ester was administrated in combination with DES-10 μg it elevated significantly T levels in blood above normal levels but this did not result in any attenuation of the DES-induced reduction in LC volume (Table 2).

Discussion

The current study was designed on detailed developmental quantitative analysis of LC volume/number and LC function (testosterone production) in rats subjected to hormonal manipulation during neonatal life using single and combined treatment with GnRHa and DES. Such treatments were already proved to cause substantial lowering of both gonadotrophin and androgen levels and elevation of estrogen levels [3]. Our results demonstrated that treatment with a potent estrogen as DES during neonatal life severely retarded LC development in a dose-dependent manner and these data are consistent with our previous results [2] for germ and Sertoli cell development. Similar negative effect on LC was induced by suppression of gonadotrophin secretion via neonatal administration of a potent long lasting GnRH-antagonist. However, both DES- and GnRHa-treated rats exhibited near normal number of LC per testis in adulthood, although functionally these cells were subnormal (as established by T levels), especially in DES-treated animals. We have previously reported [2] that LH levels in such adult animals are comparable with controls and subnormal function of adult LC was explained by permanent reprogramming of adult LC function by neonatal estrogen exposure. Discordant changes in LC numbers, function and LH levels in adulthood have also been reported for rats made hypothyroid in neonatal life [6].

The development of ALC in the rat passes through three distinct phases – PLC, ILC and ALC [4]. Each of these cell types expressed the marker enzyme 3βHSD, as well as LH-receptors, and all produce androgens but due to differences in expression of steroidogenic enzymes, they produce different androgens [4]. The delay in LC development, we found, may have resulted from inhibition of differentiation of PLC due to a direct estrogenic effect or from suppression of androgen or gonadotrophin levels [9]. Our finding that near normal numbers of LC developed in adult testis of DES treated animals suggested that the number of PLC was not adversely affected and that once DES treatment was stopped, these cells were able to enter the normal differentiation pathway. However, this interpretation does not explain why, in adulthood, LC failed to maintain normal testosterone production in the face of normal LH levels [9]. One possible explanation is that the final differentiation step of transformation of ILC into ALC could be affected resulting in an impaired ability of ALC to secrete testosterone [5].

Our recent data indicated [3] that endogenous androgens, as well FSH, exert positive effect and exogenous estrogens – negative effect on rat testis, involving germ and Sertoli cell development. The present study provides an additional means of evaluating the role of gonadotrophin, androgens and estrogens in developmental regulation of LC, as the latter express LH -, androgren receptors and estrogen receptor-α [9]. Comparative analysis of the effects of DES and GnRHa suggested if DES exerts direct estrogenic effect on LC or – indirect effect via suppression of gonadotrophin or androgen levels. In

terms of retardation of LC development and function, the effects of both single treatments with GnRHa and DES-10 μg were closely comparable at 18–25 days of age and combined treatment with DES-10 μg+GnRHa was unable to induce any greater loss of LC volume at day 18 than either treatment alone. However, in GnRHa-treated animals by day 35 there was an impressive spurt of Leydig cell development that was not seen in rats treated with DES-10 μg. Moreover, testosterone levels in adulthood were also significantly higher in GnRHa- than in DES-10 μg treated rats. The comparison of the effects of GnRHa and DES suggested that DES exerted some direct estrogenic effects on LC development that couldn't be attributed to gonadotrophin or androgen suppression. This interpretation is reinforced by our finding that treatments with low doses of DES (1 or 0.1 μg) did not produce as much as adverse effect in LC development as seen in GnRHa-treated rats.

Studies involving induction of neonatal hypothyroidism revealed that the final Sertoli cell number determines final LC number [7] as both cell types showed parallel increase in their numbers. Our previous data demonstrated a similar decrease of about 50 % in Sertoli cell number in GnRHa and DES-10μg treated rats and lesser decrements in that parameter with lower doses DES [2]. Despite this major reduction in Sertoli cell number no significant decrease in adult LC number was found in all treatment groups in the current study. This seems to rule out the possibility of a straightforward relationship between Sertoli cell and LC number in the adult testis.

Our results showed that manipulation of hormone environment during the first 2–3 weeks of postnatal life did not produce any major consistent effect on final LC number in adulthood. A possible explanation of this finding is that the factors determining ALC number are already predetermined at birth or are not dependent/sensitive to gonadotrophins, androgens and estrogens in the neonatal/pubertal period.

References

1. Abney, T. The potential roles of estrogens in regulating Leydig cell development and function: A review. – Steroids, **64**, 1999, 610-617
2. Atanassova, N., C. McKinnell, M. Walker, K.J. Turner, J.S. Fisher, M. Morley, M.R. Millar, N.P. Groome, R.M. Sharpe. Permanent effect of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenensis in adulthood. – Endocrinology, **140**, 1999, 5364-5373.
3. Atanassova, N., M. Walker, C. McKinnell, J.S. Fisher, R.M. Sharpe. Evidence that androgens and oestrogens, as well as FSH, can alter Sertoli cell number in the neonatal rat. – J. Endocrinol., **184**, 2005, 107-117.
4. Ge, R.S., L.H. Shan, M.P. Hardy. Pubertal development of Leydig cell. – In: The Leydig Cell (Eds. A.H. Payne, M.P. Hardy, L.D. Russell). Cleawater FL., Cache River Press, 1996, 159-172.
5. Ge, R.S., M.P. Hardy. Variation in the end products of androgen biosynthesis and metabolism during postnatal differentiation of rat Leydig cells. – Endocrinology, **139**, 1998, 3787-3795.
6. Habert, R., H. Lejeune, J. M. Saez. Origin, differentiation and regulation of fetal and adult Leydig cells. – Moll. Cell. Endocrinol., **179**, 2001, 47-74.
7. Hardy, M.P., R.S. Sharma, N. K. Arambepola, C. M. Sottas, L. D. Russell, D. Bunick, R. A. Hess, P. S. Cooke. Increased proliferation of Leydig cells induced by neonatal hypothyroidism in the rat. – J. Androl., **17**, 1996, 231-238.
8. Jegou, B., R.M. Sharpe. Paracrine mechanisms in testicular control.– In: Molecular Biology of the Male Reproductive System (Ed. D.M. deKretser). New York, Academic Press, 1993, 271-310.
9. Mendis-Handagama, S. M. L. C., H. B. S. Ariyaratne. Differntiation of adult Leydig cell proliferation on the postnatal testis. – Biol. Reprod., **65**, 2001, 660-671 (minireview).
10. Sharpe, R. M., M. Walker, M. R. Millar, N. Atanassova, K. Morris, C. McKinnell, P. T. K. Saunders, H. M. Fraser. Effect of neonatal gonadotropin-releasing hormone antagonist administration on Sertoli cell number and testicular development in the marmoset: Comparison with the rat. – Biol.Reprod., **62**, 1685-1693.