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The Differential Effects of the Indazole-Carboxylic Acid Derivative, Tolnidamine, on Sertoli Cell Protein Secretion

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The indazole-carboxylic acid derivative tolnidamine (TOL) has marked antispermatogenic activity in rats. Previous morphological and biochemical studies indicate that Sertoli cells are one of the targets of this compound. The aim of this study was to assess the effect of TOL on the *in vitro* secretory functions of primary Sertoli cell-enriched cultures prepared from rats of different ages by monitoring the changes of three known Sertoli cell proteins, androgen binding protein (rABP), transferrin (rTF), and testibumin (rTB). The addition of TOL at the beginning of the culture period reduced the plating efficiency of Sertoli cells; however, TOL did not induce a significant change in cell number if it was added 24 h after plating of the cells. Sertoli cell-enriched cultures prepared from testes of 10-day-old rats were highly sensitive to TOL as evidenced by a marked inhibition in secretions of rABP, rTB, and rTF in all experiments. In cultures prepared from 15- and 20-day-old rats, TOL had no apparent effect on rABP secretion, but reduced rTF and increased rTB secretion. Thus, TOL has a differential effect on the secretion of individual proteins in Sertoli cells cultured from rats between 10 and 20 days of age. This phenomenon is presumably a consequence of the progressive maturation of Sertoli cells in the seminiferous epithelium.

Key words: Indazole-carboxylic acids, tolnidamine, antispermatogenic agents, Sertoli cell, androgen binding protein, testibumin, transferrin

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Indazole-carboxylic acid derivatives have been shown to possess marked antispermatogenic activity in several animal species; thus, their usefulness in fertility control has been implicated (Burberi et al, 1975; Coulston et al, 1975; Silvestrini et al, 1975; 1978; Corsi et al, 1976; Lobl et al, 1979; Cioli et al, 1980; James et al, 1980). Several studies have shown that the administration of one such derivative, tolnidamine, 1-(4-chloro-2-methyl-benzyl)-1H-indazole-3-carboxylic acid (TOL), to rats at doses higher than required to induce an antispermatogenic effect will produce a minimal and transient increase in serum concentrations of LH and FSH without alteration in testosterone levels (Lobl et al, 1981; James et al, 1980). Morphological studies after treatment with TOL and other indazole-carboxylic acid derivatives have shown that these agents produced a rapid and dramatic effect on the seminiferous tubular epithelium in the rat, rabbit, dog, and monkey (Lobl et al, 1979; Buthala and Lobl, 1979; De Martino et al, 1981; Francavilla et al, 1986). Since Sertoli cells are a major secretory component of this epithelium, it is pertinent to study the pharmacological effects of tolnidamine on Sertoli cell

secretory activity *in vitro* using three known Sertoli cell proteins, rABP, rT, and rTB.

Materials and Methods

Preparation of Primary Sertoli Cell-Enriched Cultures

Sprague-Dawley male rats of 10, 15, and 20 days of age were obtained from Charles River Laboratories (Kingston, MA). Primary Sertoli cell-enriched cultures were prepared using established procedures (Mather and Phillips, 1984). Groups of 20–40 animals were used for each set of experiments to provide adequate cell numbers. Sertoli cell aggregates were seeded on culture plates at a density of approximately $1–3 \times 10^6$ cells/dish. The culture medium was prepared from Ham's F-12 Nutrient Mixture and Dulbecco's Modified Eagle's Medium (F12/DME) (1:1, v/v) with the addition of 1.2 g/liter sodium bicarbonate, 15 mM Hepes, and 20 mg/liter gentamycin, and supplemented with insulin (10 μg/ml), human transferrin (5 μg/ml), and epidermal growth factor (2.5 ng/ml). The cells were cultured at 35C in a humidified atmosphere of 5% CO_2/95% air (v/v). Cell cultures were examined with a Nikon inverted microscope using phase optics (Nikon Inc., Garden City, NY). Each experiment was repeated at least twice.

Treatment of Cells with TOL—Effects of a Single Concentration of TOL on Sertoli Cell Secretory Activities

Primary Sertoli cell-enriched cultures prepared from 10-, 15-, and 20-day-old rats were treated with TOL (1 μg/ml) at the time of plating. The cells were maintained in the same medium for up to 6 days. At the end of each day, a set of six dishes (three dishes from each treatment regimen) was terminated and the media were collected and stored at −20°C until used. The number of individual cells attached to each culture dish was determined by removing cells from the plate with 0.1% trypsin (v/v), neutralizing with an equal volume of fetal calf serum-supplemented (7.5%, v/v) F12/DME medium, and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL).

Effects of Different Concentrations of TOL on Protein Secretion by Sertoli Cell-Enriched Cultures

Primary Sertoli cell-enriched cultures prepared from rats at 10, 15, and 20 days of age were plated on dishes for 24 h. Thereafter, the media were removed and TOL was added at concentrations between 1, 10, 100, and 1000 ng/ml. The media were changed after 3 days and the same amounts of TOL were added to each dish. On Day 7, the media were collected, the amounts of rABP, rTF, and rTB were determined by respective radioimmunoassays, and the number of cells in each dish was determined. As set of three dishes was used for each experiment group. The cell number was determined in the same well where the media were collected as described above.

Radioimmunoassays

rTB was measured by RIA using procedures previously described (Cheng and Bardin, 1986). The rTB standard curve was calibrated using a pool of Sertoli cell-enriched culture medium (EP125-130) derived from Sertoli cells grown for 8 days in the presence of both FSH (300 ng/ml) and testosterone (1×10^{-7}M). The minimal detectable dose was 0.4 μl eq/assay tube, and 50% displacement was at 5 μl eq (1 μl eq of EP125-130 equivalent to 3.8 ng of purified testibumin). The intra-assay coefficient of variation was 8%, and the interassay coefficient of variation was 15% (Cheng and Bardin, 1986).

rABP was measured by RIA and expressed as microliter equivalents μl eq of a standard epididymal cytosol (GMB-E-1), which was run in every assay as previously described (Gunsalus et al, 1978a, 1978b). Comparison of GMB-E-1 with the reference preparation WR-R1 from the NIH showed that 1 μl eq of GMB-E-1, was equivalent to 9.8 ± 0.9 fmole of binding sites. The intra-assay coefficient of variation was 8%, and the inter-assay coefficient of variation was 16%. The minimum detectable dose was 0.1 μl eq. and 50% displacement was at 1.3 μl eq.

rTF in culture media was measured by RIA as previously described (Perez-Infante et al, 1986) and expressed as ng of a reference standard using purified rat transferrin. The minimum detectable dose was 0.1 ng per assay tube. The intra-assay coefficient of variation was 4%, and the inter-assay coefficient of variation was 15%.

All samples of a given experiment were measured in a single RIA to eliminate inter-assay variations. Each standard curve was run in triplicate and the unknown samples in duplicate. All assays were computer analyzed by fitting standard curves to a four-parameter logistic function and interpolating unknowns from the resulting curve using a program adapted from Rodbard et al (1975).

General Methods and Statistical Analysis

Results were analyzed using the BMDP statistical software (Dixon et al, 1983). One-way analysis of variance (BMDP7D) was used to determine mean statistical differences between various groups. Cell viability was monitored by trypan blue staining (0.4%, w/v) in 0.81% NaCl and 0.06% dipotassium phosphate.

Results

Effect of TOL on Protein Secretion by Cultures Prepared from 10-, 15- and 20-day-old Rats

For 10-day-old rats: in the absence of TOL, the number of cells in the control dishes remained stable during the first 4 days in culture, but decreased by two-thirds on Days 5 and 6 ($p < 0.001$ compared to Day 1) (Fig. 1A). The addition of TOL at the beginning of culture reduced the number of cells plated on each culture dish ($p < 0.001$ compared to the controls); however, the cell number remained constant thereafter and by Day 5 was the same as in the controls (Fig. 1A). Cell viability at the end

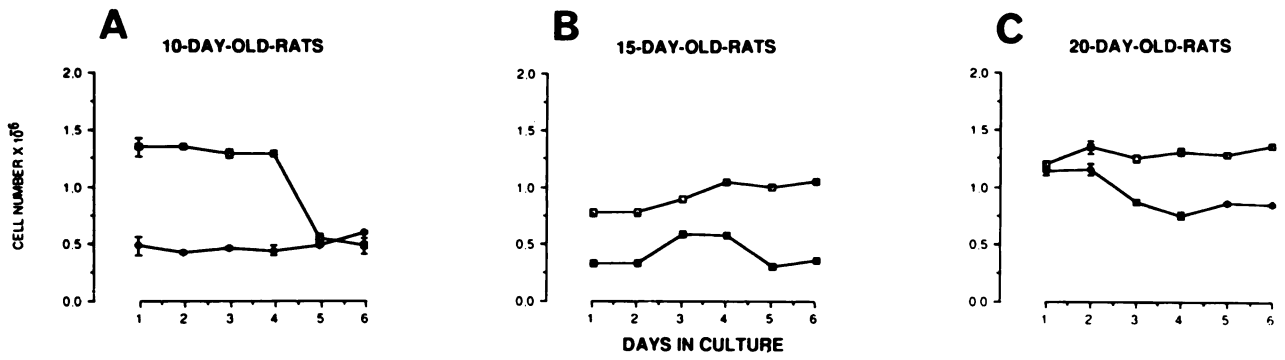

Fig. 1. The effect of TOL on the number of Sertoli cells *in vitro*. Sertoli cell number in culture was determined at a 24-h interval using a Coulter Counter following trypsin treatment as described in Materials and Methods. Sertoli cell-enriched cultures were prepared from rats at 10 (A), 15 (B), and 20 (C) days of age using established procedures as described under Materials and Methods. (⊡) controls (●) TOL 1 μg/ml. Data are expressed as mean ± SEM of three separate (n = 3) culture dishes for each time point.

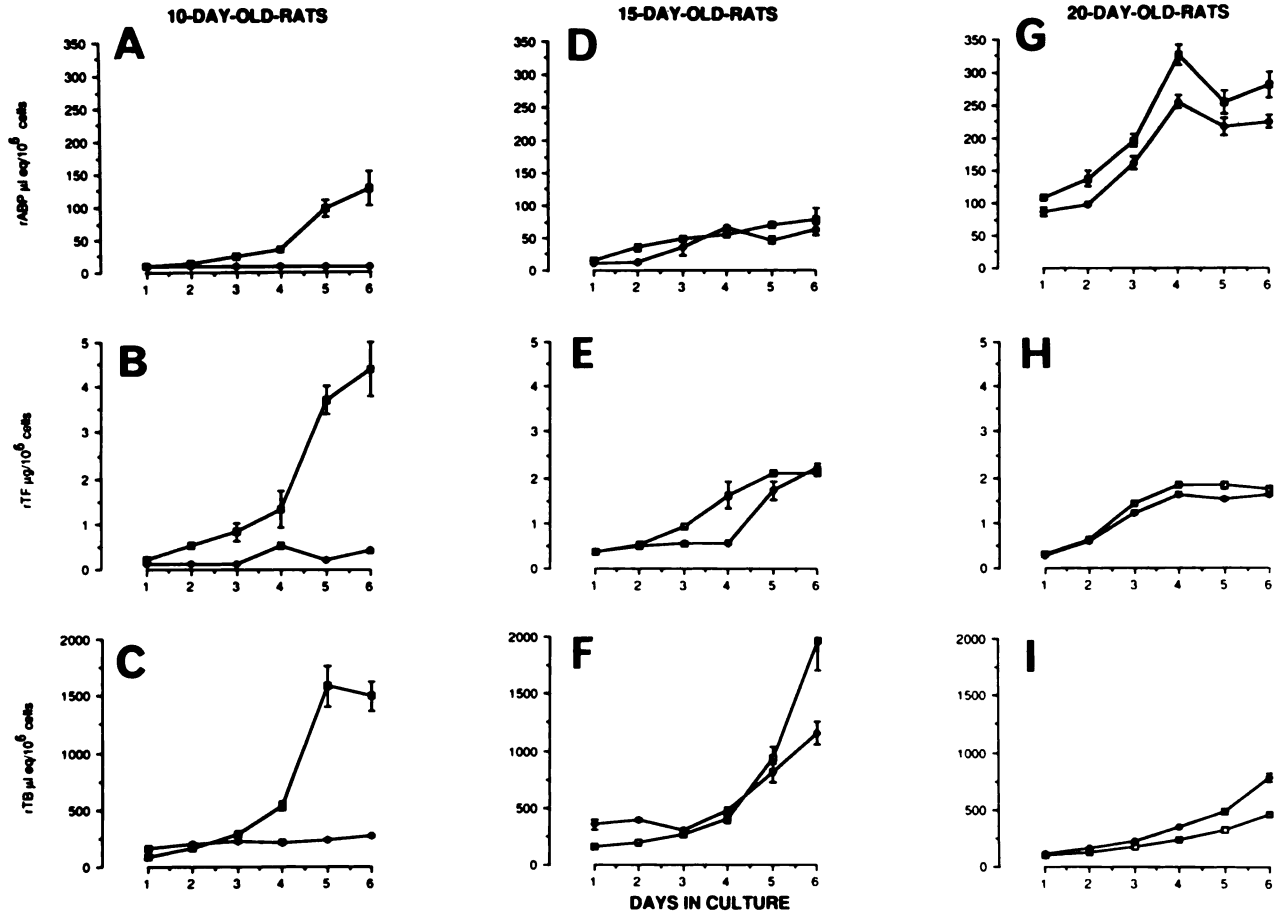

Fig. 2. Effects of TOL on the secretions of rABP (A, D, G), rTF (B, E, H), and rTB (C, F, I) by Sertoli cells *in vitro*. Sertoli cell-enriched cultures were prepared from rats at 10, 15, and 20 days of age treated either with 1 μg/ml TOL (●), or without TOL (⊡). Values are the cumulative mean ± SEM from triplicate culture. Media were collected in corresponding time points and the amount of Sertoli cell proteins was estimated by respective RIAs.

of each experiment was monitored by trypan blue staining, in which dead cells were stained. It was noteworthy that the number of viable cells in each control and treated group was greater than 95% at the end of the experiment. In view of the alterations in cell number in each treatment regimen, the secretory function of Sertoli cells was expressed in terms of cell number instead of per ml media.

The secretion of rABP, rTB, and rTF by Sertoli cell-enriched cultures increased with time. The addition of TOL significantly inhibited the secretion of rABP, rTB, and rTF beginning at Day 2–4 of culture ($p < 0.001$) and this inhibition persisted until the end of the culture period on Day 6 (Fig. 2A,B,C).

For 15-day-old rats: the cell number in control dishes without TOL remained relatively constant throughout the entire culture period (Fig. 1B), which is strikingly different than cultures prepared from rats at 10 days of age (Figs. 1A and 1B). The addition of TOL in the beginning of the culture period, however, significantly reduced the number of Sertoli cells attached per dish, similar to cultures prepared from rats at 10 days of age (Fig. 1B). Treatment of cultures with TOL produced no consistent changes in the secretion of rABP (Fig. 2D); decreased rTF secretion on Days 3 and 4 (Fig. 2E); and increased rTB secretion during the first 2 days, although its secretion was reduced by Day 6 ($p < 0.01$) (Fig. 2F).

For 20-day-old rats: in control cultures, the cell number remained relatively constant throughout the entire culture period (Fig. 1C). The addition of TOL at the beginning of the culture period did not change the number of cells plated per dish. However, by Day 3 of culture, TOL significantly decreased the cell number ($p < 0001$), which remained relatively constant thereafter (Fig. 1C).

TOL did not produce any significant changes on rTF secretion by Sertoli cell-enriched cultures (Fig. 2H). It decreased rABP (Fig. 2G), but stimualted rTB secretion (Fig. 2I).

Dose Response Effects of TOL on Protein Secretion

The addition of different concentrations of TOL to Sertoli cell-enriched cultures after the cells were allowed to attach for 24 h, did not induce any consistent changes in cell numbers in any of the cultures examined when compared with the controls (Fig. 3A). The cell numbers, however, were higher in cultures prepared from rats at 15 and 20 days of age (Fig. 3A). In cultures prepared from 10-day-old rats, all doses of TOL decreased both rABP (Fig. 3B) and rTB (Fig. 3D) secretion ($p < 0.001$); whereas

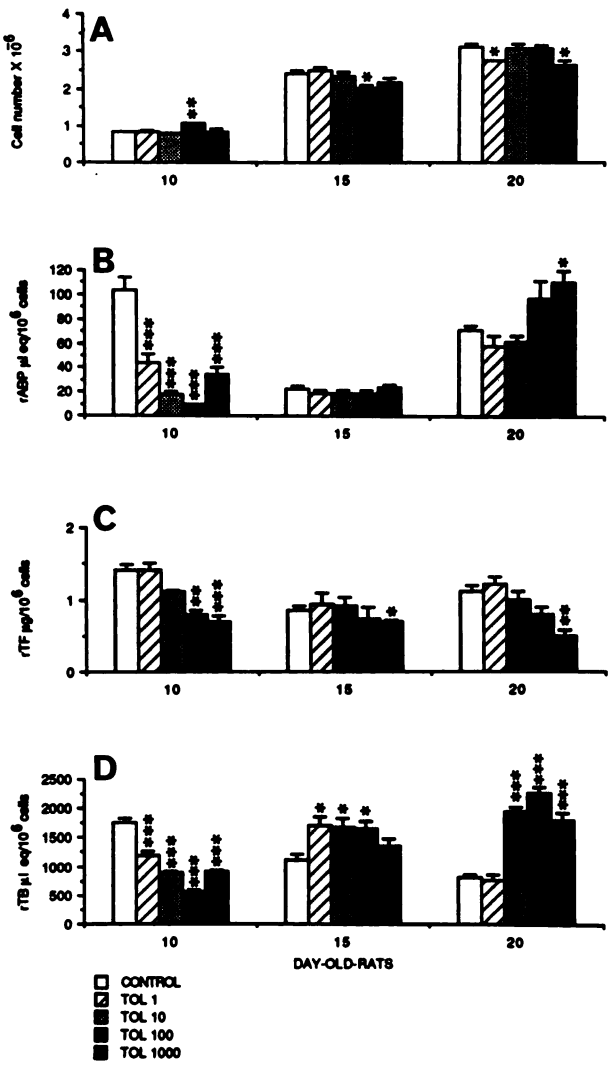

Fig. 3. Effect of different concentrations of TOL on cell number (A), and the secretions of rABP (B), rTF (C), and rTB (D) by Sertoli cell-enriched cultures prepared from rats at 10, 15 and 20 days of age. Primary Sertoli cell-enriched cultures prepared from rats of different ages were plated for 24 h, media were removed, and appropriate concentrations of TOL were added. Media were changed at the end of 3 days of culture and fresh media along with TOL were added. On Day 7, media were collected and the concentrations of rABP, rTF, and rTB were measured by radioimmunoassay. Each bar represents the mean ± SEM of six cultures. Asterisks indicate the significance levels for the multiple comparison between TOL and controls (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

rTF secretion was decreased only at higher doses ($p < 0.01$) (Fig. 3C). In cultures prepared from 15-day-old rats, TOL had no effect on rABP, decreased rTF secretion at a dose of 1000 ng/ml, and stimulated rTB secretion ($p < 0.05$) at doses between 1 and 100 ng/ml (Fig. 3B,C,D). In cultures from 20-day-

old rats, TOL induced a slight increase in rABP secretion ($P < 0.05$), decreased rTF, and increased rTB secretion at doses between 10 and 1000 ng/ml ($p < 0.001$) (Figs. 3B,C,D).

Discussion

It is noteworthy that the number of Sertoli cells in cultures derived from rats at 10 days of age without added TOL decreased with time in culture in a pattern consistent with previously published results (Rich et al, 1983). Such a decrease in cell number following *in vitro* culture was not observable in cells prepared from 15- and 20-day-old rats. The immediate explanation for this observation is not known; however, these data are in agreement with previously published results (Rich et al, 1983). It is noteworthy that the cell number appears to increase in cultures prepared from 15-day-old rats, suggesting that cell division might have occurred in these cultures.

The present study examined the effects of various doses of TOL on the secretory functions of Sertoli cells in culture by monitoring the changes of three Sertoli cell secretory proteins. The results show that there are differential effects of TOL on the secretion of rABP, rTB, and rTF by Sertoli cell-enriched cultures *in vitro*, and that the magnitude and the responses are dependent on the age of the animals and the time of the addition of TOL into the cultures.

TOL, when added to the culture medium at time 0, produced a reduction in plating efficiency in cultures prepared from 10- and 20-day-old rats. In contrast, when TOL was added 24 h after the cells were seeded, there were no significant alterations in cell number. Sertoli cell-enriched cultures prepared from 10-day-old rats were highly sensitive to TOL treatment, which consistently inhibited the secretion of rABP, rTB,and rTF. This effect was evident whether or not the culture media were changed during the culture period. By contrast, in cultures prepared from 15- and 20-day-old rats, TOL induced no apparent effects on rABP secretion, decreased rTF, and increased rTB secretion; however, such inhibitory and stimulatory effects were not as evident as cultures prepared from 10-day-old rats. These findings suggest that such differential responses of Sertoli cells to TOL treatment might be related to the maturation status of the Sertoli cells.

It was noteworthy that TOL increased rTB secretion in cultures prepared from 15- and 20-day-old rats, whereas it significantly inhibited its secretion in cultures prepared from 10-day-old-rats. This result could be due to increased secretion, decreased proteolysis, or a combination of both. Regardless of the mechanism, the result must be considered a specific effect of TOL activity since, in the same cultures, TOL produced no apparent change in the accumulation of rABP and rTF.

An *in vivo* study is in progress to ascertain if the administration of an antispermatogenic dose of TOL to adult rats produces parallel changes in serum, testicular, and epididymal rTB levels, and to correlate the alteration of Sertoli cell secretory function observed *in vivo* and *in vitro*.

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