The Effects of the Indazole Carboxylic Acid Derivative, Tolnidamine, on Testicular Function: I.

Early Changes in Androgen Binding Protein Secretion in the Rat

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The indazole carboxylic acid derivative, tolnidamine, has marked antispermatogenic activity in several animal species. In this study, we assessed the effect of tolnidamine on rat Sertoli cell function both *in vivo* and *in vitro*, using androgen binding protein (rABP) as a marker. Groups of six male rats were killed 2, 4, 8, 16, 32, 64 hours and 5, 8, and 12 days following tolnidamine administration (250 mg/kg by oral gavage). There was a progressive reduction in both testicular and epididymal weights. Serum FSH levels did not change and LH showed a transient increase between 64 hours and 8 days. Except for an initial increase at 2 hours, there were no changes in serum testosterone. Epididymal rABP concentration and content declined as early as 8 hours, with the lowest values occurring at 5 and 12 days. By 16 hours, there was an increase in testicular rABP, which was also evident at 8 days and 12 days. Within 16 hours after tolnidamine, there was a rise in serum rABP, which persisted until the end of the experiment. When another indazole carboxylic acid derivative, lonidamine, was administered (250 mg/kg), similar changes were evident in epididymal and serum rABP at 32 hours, but the rapid decrease in testicular rABP suggested a different mechanism of action. In another experiment, single oral doses of tolnidamine (50, 100, 250, and 500 mg/kg) were administered to other groups of rats and the animals were killed after 24 hours and 5 days. With increasing doses of tolnidamine, there was a reduction in epididymal rABP concomitant with an increase in testis and serum rABP levels. Since rABP levels in the testis and epididymis change following treatment with tolnidamine, we wished to determine whether this agent had a direct effect on Sertoli cells; this possibility was investigated *in vitro*. Sertoli cells were prepared from 20-day-old animals and cultured in serum-free medium containing insulin, transferrin, epidermal growth factor, and various concentrations of lonidamine or tolnidamine. By 7 days, there were no significant effects on rABP secretion into the medium with either agent. It can be concluded that a single dose of tolnidamine has a minimal effect on the pituitary Leydig cell axis since there was only a transient increase in serum LH with no alterations in testosterone. In contrast, this drug had a dramatic and rapid effect on seminiferous tubule function as manifested by disruption of the dynamics of rABP secretion. The reduction in epididymal rABP may be best explained by inhibition of the passage of tubular fluid to the epididymis. Obstruction of efferent ductules was unlikely because there was no increase in testicular weight. The increase in testicular rABP was presumably related to accumulation secondary to reduced secretion into, or transport from, the seminiferous tubular lumen. The rise in serum rABP is best explained by increased testicular rABP concentration. The failure to observe a change in rABP concentration during incubation of cultured Sertoli cells with increasing doses of tolnidamine implies a lack of a direct effect of tolnidamine on rABP synthesis, and suggests that this drug primarily alters the kinetics of secretion from the intact tubule.

Key words: tolnidamine, rABP; LH, FSH, testosterone; Sertoli cell.

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Three of the indazole-3-carboxylic acid derivatives, AF 1312/TS, lonidamine (AF 1890) and tolnidamine (AF 1923), have marked antispermatogenic activity in mice, rabbits, dogs, and monkeys (Coulston et al, 1975; Silvestrini et al, 1975; DeMartino et al, 1975; 1981; Lobl et al, 1979). Since these agents appear to have direct actions on the testicular germinal epithelium of these species, they hold promise as potential male contraceptive agents (Silvestrini, 1981). High doses of these agents produce irreversible sterility. At low doses, however, there may be partial or complete restoration of spermatogenesis when the

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drug is discontinued (Lobl, 1979; Coulston, 1981; Silvestrini, 1981). Preliminary results have suggested that spermatogenesis in monkeys may be reversibly controlled by once-a-week oral administration of AF 1312/TS (Coulston, 1981).

Studies have therefore been conducted to understand the mechanism of action of indazole carboxylic acids. When given to the intact animal, [^{3}H] lonidamine rapidly disappeared from all tissues and did not concentrate in the reproductive system, suggesting that the antifertility effects did not depend on its continuing presence (Lobl, 1979). Studies in the rat have shown that lonidamine produced a rapid rise of the Sertoli cell product, androgen binding protein (rABP), in serum, which was accompanied by a decrease in rABP in both the testis and epididymis (Lobl et al, 1981). Since tolnidamine appears to be less toxic than lonidamine (Silvestrini, 1981), we examined its effects on rat ABP kinetics. In the present study, we demonstrate that, while tolnidamine also rapidly changes the concentrations of rABP in serum, testis, and epididymides, the resulting pattern is strikingly different from that produced by lonidamine.

Materials and Methods

Materials

Tolnidamine [1-(4-chloro-2-methylbenzyl)-1H-indazole-3-carboxylic acid] was supplied to us by Dr. B. Silvestrini, Instituto di Ricerca F. Angelini, Rome, Italy, and lonidamine [1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid] by Dr. R. Blye, Contraceptive Development Branch, Center for Population Research, National Institute of Child Health and Human Development.

Animals and Treatment

Male Charles River rats were housed under a schedule of 14 hour light: 10 hour darkness, and water and rat chow were available *ad libitum*. All treated animals received oral doses of tolnidamine suspended in 0.25% methyl cellulose. Control animals were given vehicle alone.

A longitudinal study was performed in 90-day-old animals that received a single oral dose of tolnidamine (250 mg/kg). Animals in groups of six were then killed by decapitation at 2, 4, 8, 16, 32, 64 hours, and on the 5th, 8th, and 12th day following tolnidamine administration. Except for the first three time periods, all animals were killed between 8:30 and 10:30 a.m. For the initial time courses, the drug was administered at 8:30 a.m. and the animals killed after 2, 4, or 8 hours, respectively. For purposes of comparison, groups of rats received lonidamine (250 mg/kg), and were killed at 4 and 32 hours. Two control groups of rats were administered methyl cellulose only, and were also killed after 4 and 32 hours.

In a second experiment, 90-day-old animals received a single dose of either vehicle or tolnidamine (50, 100, 250, and 500 mg/kg). For each dose regimen, groups of six rats were then killed at 24 hours and at 5 days.

In both experiments, blood was collected after decapitation, centrifuged, and the serum stored at -20 C until assayed. For rABP measurement, testicular and epididymal cytosols were prepared from a single testis and epididymis as previously described and stored at -20 C until assay (Vogel et al, 1983).

Primary Sertoli Cell Cultures

Sertoli cells were isolated from testes of 20-day-old Sprague-Dawley rats and cultured for periods up to 7 days in serum-free medium supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), and epidermal growth factor (2.5 ng/ml), as previously described (Rich et al, 1983). Concentrations of tolnidamine or lonidamine, ranging from 25 ng/ml to 2.5 μg/ml, were added to the culture medium. The medium was changed every 3 days. The number of individual cells attached to the culture dish on day 7 was determined by removing the cells from the plate and counting with a Coulter counter. Cell numbers were determined in duplicate for each of the drug concentrations. Rat ABP secreted by Sertoli cells into the medium over the last 72 hours of the experiment was determined by radioimmunoassay. The complete experiment was performed on two separate occasions.

Radioimmunoassays

Plasma LH and FSH were measured by double antibody radioimmunoassays using reagents supplied by the National Pituitary Agency, NIAMDD (Goldstein et al, 1983). Results were expressed in ng/ml NIAMDD rat LH-RP-1 and FSH-RP-1 standards supplied with the kit. Inter-assay coefficients of variation for LH and FSH assays were 16.6% and 13.1%, respectively. Corresponding intra-assay coefficients of variations were 12.1% and 8.2%, respectively. For the testosterone assay, plasma samples were initially extracted with ethyl ether and then assayed (Goldstein et al, 1983). The cross-reaction of the anti-testosterone antibody with dihydrotestosterone was 24%. Bound and free testosterone were separated by the dextran-coated charcoal technique.

Androgen binding protein was isolated from rat epididymides, as previously described (Musto et al, 1977; 1980), and used to prepare antiserum and assay reagents. Serum, testicular, and epididymal samples were assayed by radioimmunoassay (Gunsalus et al, 1978; Vogel et al, 1983). All LH, FSH, testosterone, and rABP determinations from each experiment were measured at the same time.

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 the various groups. When appropriate, the Student's *t*-test (BMDPIV) was used to compare the response in tolnidamine-treated rats with their corresponding controls.

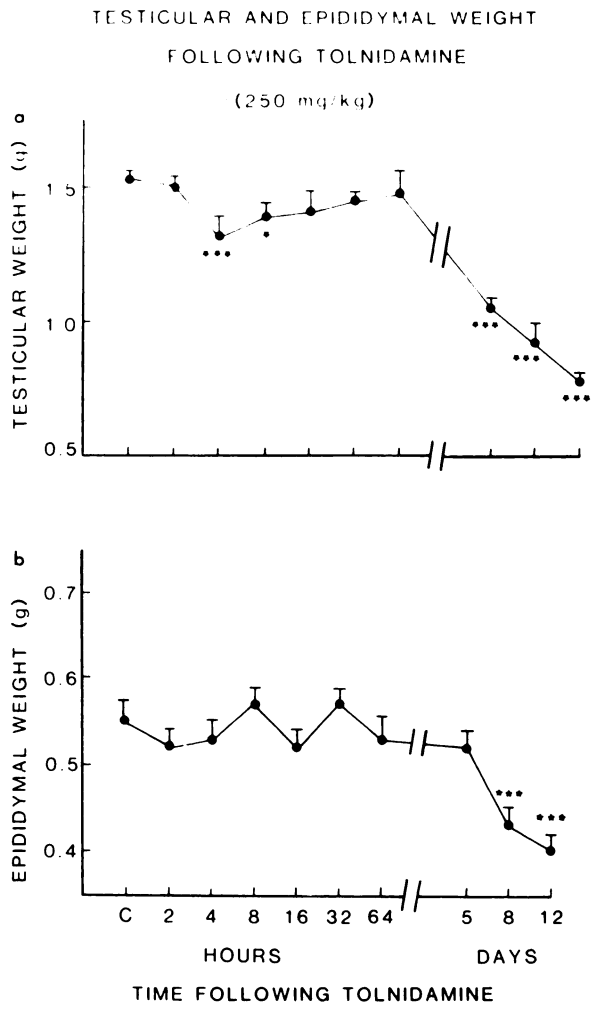
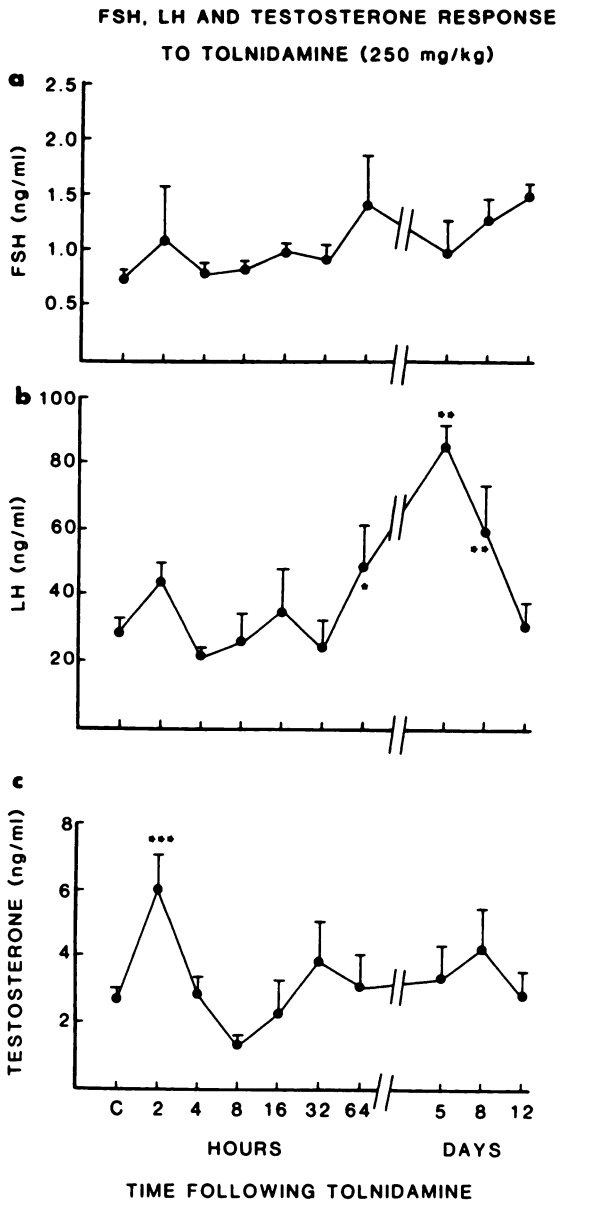


Fig. 1. The testicular (Fig. 1a) and epididymal (Fig. 1b) weights at various time intervals following administration of tolnidamine (250 mg/kg) by oral gavage. Each point represents the mean ± SEM of six rats. Twelve control rats (C) were given vehicle alone: six were killed at 4 hours and six at 32 hours. The mean ± SD epididymal weights in the two control groups were 0.55 ± 0.055 and 0.65 ± 0.08 g, respectively ($P < 0.05$). Only the 4-hour value has been plotted. Since no differences in testicular weight between the control groups were observed, the results were pooled. Asterisks indicate significant differences from control: $^{*}P < 0.05$; $^{***}P < 0.001$.



Figs. 2a, b, and c. The FSH, LH, and testosterone response to tolnidamine (250 mg/kg). Each point represents the mean ± SEM of six rats. Twelve control rats were given vehicle alone: six were killed at 4 hours and six at 32 hours. Since there were no differences between the two control groups, the results were pooled.

Results

The Effects of a Single Dose of Tolnidamine

Testicular and Epididymal Weight. Both testicular and epididymal weights decreased following tolnidamine administration (Fig. 1). There was a transient decrease in testicular weight at 4 to 8 hours, with subsequent recovery. The most dramatic effects were seen at later time periods, with a one-third reduction in testicular weight by 5 days and in epididymal weight by 12 days.

Serum Gonadotropins and Testosterone Levels. A transient rise in FSH, LH and testosterone levels occurred 2 hours following tolnidamine administration, but only testosterone levels were significantly increased ($P < 0.001$). For the remainder of the experiment, testosterone levels were not different from the controls. Analysis of variance indicated a significant transient increase in the concentration of serum LH between 64 hours and 8 days. Levels returned to

TABLE 1. Mean ± SD Epididymal, Testis and Serum rABP Following Lonidamine and Tolnidamine Administration

		Epididymis		Testis		Serum
Drug	Time (hrs)	organ content μl eq/epid$\times 10^{-2}$	tissue conc. μ eq/g $\times 10^{-2}$	organ content μl eq/testis $\times 10^{-2}$	tissue conc. μl eq/g $\times 10^{-2}$	μl eq/ml $\times 10^{-2}$
Lonid*	8	77.0 ± 24.0	135 ± 25.0	35.9 ± 3.36	24.8 ± 1.8	2.62 ± 0.52
Lonid	32	48.0 ± 5.0[a]	85.5 ± 11.5[a]	22.0 ± 7.3[b,d]	13.8 ± 4.2[b,d]	9.33 ± 1.63[a]
Tolnid†	8	58.0 ± 7.5[a]	104 ± 22.0[c]	32.1 ± 9.84	22.8 ± 6.12	3.20 ± 0.82
Tolnid	32	55.5 ± 13.0[a]	97.0 ± 19.0[a]	34.9 ± 12.3	23.9 ± 7.8	8.73 ± 2.86[a]
Controls	—	83.5 ± 12.0	141 ± 27.0	33.8 ± 6.36	22.1 ± 3.96	2.58 ±0.61

*Lonid = lonidamine.
†Tolnid = tolnidamine.
[a]$P < 0.001$ compared with controls.
[b]$P < 0.005$ compared with controls.
[c]$P < 0.05$ compared with controls.
[d]$P < 0.05$ compared with tolnidamine.
Each group consisted of six rats, except for the controls. The latter comprised 12 rats, six of which were killed 4 hours, and the remainder 32 hours, after vehicle administration.

baseline by 12 days. In contrast, serum FSH did not change during the 12 days of the study (Fig. 2).

rABP Levels in Serum, Testis, and Epididymis. The first change in rABP after tolnidamine treatment occurred in the epididymis. When expressed either as tissue concentration or organ content, there was a steady and progressive decline in epididymal rABP, which was apparent at 8 hours. The lowest values observed were at 8 and 12 days (Fig. 3a).

Within 8 hours after the first change in epididymal rABP, there was a significant rise in testicular rABP content. This and subsequent changes in rABP were significant when results were expressed as tissue concentration or as organ content. Following the peak at 16 hours, there was a transient decline, after which the levels rose again. Because of the decrease in testicular weight, this second rise in rABP was more striking when the rABP results were expressed as tissue concentration (Fig. 3b).

Sixteen hours after tolnidamine administration, there was a significant rise in serum rABP levels, which persisted until the end of the experiment (Fig. 3c). The peak occurred at 32 hours, followed by a transient decline, and the levels subsequently increased again at 8 days. At 12 days, serum rABP values were still greater than in the basal pretreatment samples (Fig. 3c).

Comparison of Tolnidamine and Lonidamine

Two groups of animals were treated with 250 mg of these drugs and killed at 8 and 32 hours (Table 1). When compared with controls, 32 hours after both lonidamine and tolnidamine administration, there was a significant increase in serum rABP as well as a reduction in rABP epididymal concentration and content (Table 1). In contrast to tolnidamine, however, lonidamine administration produced a significant reduction in testis rABP tissue concentration and organ content at 32 hours (Table 1).

The Effects of Different Doses of Tolnidamine

Testicular and Epididymal Weight. Single oral doses of tolnidamine ranging from 50 to 500 mg/kg produced no changes in testicular weight by 24 hours. The two highest doses (250 and 500 mg/kg) were associated with a significant decrease in testicular weight by 5 days ($P < 0.01$). No effect on epididymal weight was produced by any dose of tolnidamine at either 24 hours or 5 days.

Epididymal, Testicular and Serum rABP Levels. There was a trend for reduction in epididymal rABP content with increasing doses of tolnidamine, and values were significantly less than in the controls at 24 hours with tolnidamine doses of 250 mg/kg and 500 mg/kg. The most dramatic decrease, however, occurred at 5 days with 500 mg/kg tolnidamine (Fig. 4a). With respect to testicular content and concentration of rABP, significant increases occurred with all doses of 100 mg/kg or greater at 5 days. No changes were seen after 24 hours (Fig. 4b). There was a significant increase in serum rABP within 24 hours following 250 and 500 mg/kg tolnidamine; by 5 days, increases in serum rABP were evident with doses of tolnidamine of 100 mg/kg or greater (Fig. 4c).

The Direct Effects of Tolnidamine and Lonidamine on Sertoli Cells In Vitro

Since testicular rABP levels increased with tolnidamine administration and decreased with lonidamine, we wished to determine whether these agents had a differential effect on Sertoli cells *in vitro*. Cultures were prepared from 20-day-old animals and plated in

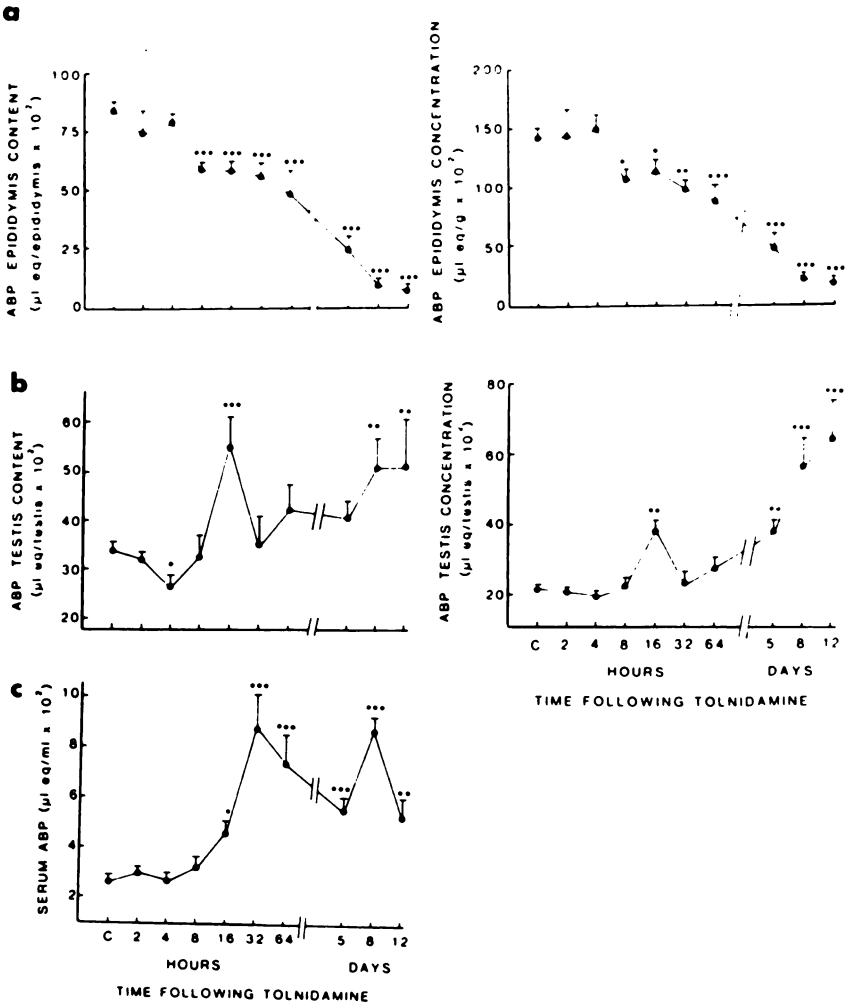


Fig. 3. The change in rABP after tolnidamine (250 mg/kg). See legend of Fig. 2 for details. Fig. 3a shows epididymal rABP content (μl eq/epididymis $\times 10^{-2}$) and concentration (μl eq/g $\times 10^{-2}$). Fig. 3b depicts testis rABP content (μl eq/testis $\times 10^{-2}$) and concentration (μl eq/testis $\times 10^{-2}$), Fig. 3c shows serum rABP μl eq/ml $\times 10^{-2}$).

supplemented medium as described above. On day 1, the medium was changed and the assigned concentrations of tolnidamine or lonidamine added. Medium was changed on day 4 and collected, and cell numbers were obtained on day 7 of culture. There appeared to be a reduction in cell number at all doses of tolnidamine, but this was not significant (Fig. 5). There was also no significant effect of tolnidamine on rABP secretion per viable cell at any dose used. Similar effects were also produced by lonidamine (Fig. 5). The same results were obtained when the experiment was repeated.

Discussion

Morphologic studies have shown that indazolecarboxylic acid derivatives similar to those in the present report affect both Sertoli cells and mature germ cells without apparent damage to spermatogonia or to the interstitial tissue (Silvestrini et al, 1975; DeMartino et al, 1975; 1981). The sensitivity of the testes to these agents depends on the species, the dose, and duration of treatment. Rat testes are the most sensitive; mouse, dog and rabbit require higher doses administered for a longer time period to produce changes (DeMartino et al, 1975; 1981). The earliest morphologic lesions are characterized by vacuolization and apical retraction of Sertoli cell cytoplasm, which results in the release of immature germ cells into the tubular lumen (DeMartino et al, 1975; 1981). For this reason, these drugs are known as exfoliative antispermatogenics. Among the different germ cells, those at a more mature stage of spermatogenesis, ie, spermatids and spermatocytes, are more sensitive to the drugs (DeMartino et al, 1981; Marcante et al, 1981).

In the present study, there was a transient increase in LH, but no consistent alterations in FSH or testosterone levels. In contrast, slight increases in FSH, but not LH or testosterone, have been observed previously in the rat with large doses of both lonidamine

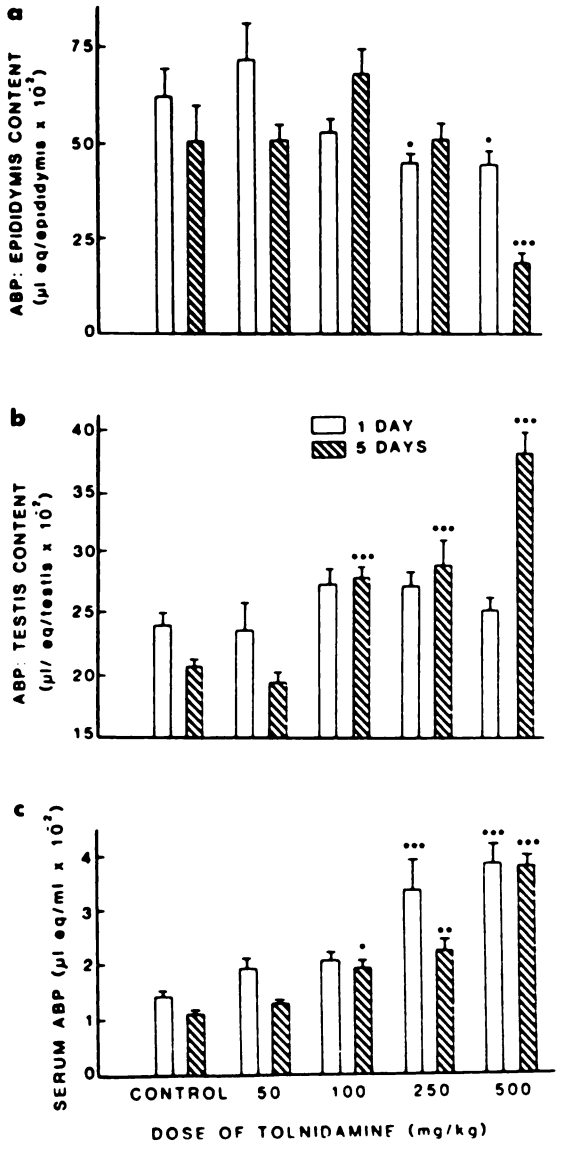


Fig. 4. The change in rABP after 50, 100, 250, and 500 mg/kg. One group of animals was killed at 24 hours (open bars) and another group at 5 days (stripped bars). Fig. 4a shows the epididymal rABP content (µl eq/epididymis × 10^{-2}), Fig. 4b the testis rABP content (µl eq/testis × 10^{-2}), and Fig. 4c the serum rABP (µl eq/ml × 10^{-2}). Values represent the mean ± SEM of six rats. The two groups of control animals received the vehicle alone and were killed at the same time intervals. Asterisks indicate significant differences between tolnidamine-treated animals and the corresponding controls: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$. There were no significant differences between the amounts of rABP in epididymides, testes, and serum of the two groups of controls (24 hours and 5 days). Since rABP organ contents and tissue concentrations showed the same trend as in Fig. 3, only the former have been depicted.

and tolnidamine (Lobl, 1979; Lobl et al, 1981). The reasons for these differences are not apparent. In the monkey, neither gonadotropins nor testosterone change (Lobl et al, 1979). It can thus be concluded that single doses of the indazole carboxylic acids do not produce major alterations in gonadotropins or testosterone. Consistent with this conclusion was the failure to observe alterations in ventral prostate weight upon short-term drug administration (Lobl, 1979). On the other hand, chronic lonidamine therapy over several weeks in the rat was associated with reduction in seminal vesicle weight (Lobl, 1979).

The present study demonstrated a steady decline in testicular weight, which is in agreement with previously reported results with this as well as the other derivatives (Coulston et al, 1975; Silvestrini et al, 1975; Lobl, 1979; Lobl et al, 1981).

In the rat, rABP has been used extensively to study Sertoli cell and seminiferous tubular function *in vivo* and *in vitro* (Bardin et al, 1981). In the intact animal, 80% of this protein is secreted from the apex of Sertoli cells in the tubular lumen from whence it is transported to the epididymis; the remainder is secreted from the base of Sertoli cells into the blood and lymphatics of the interstitial space (Gunsalus et al, 1980; 1981; Mather et al, 1983). According to these observations, rABP is secreted bi-directionally from Sertoli cells. Recent observations suggest that there is even a qualitative difference in the rABP secreted into the tubular lumen and into the blood (Cheng et al, 1984). A variety of studies indicate that hormones, drugs, and testicular disease can selectively alter the apical secretion of rABP (into the tubular lumen) independent of basal secretion (into the interstitial space). An understanding of this bi-directional secretion of rABP by Sertoli cells permits us to formulate a working hypothesis concerning the differential actions of lonidamine and tolnidamine.

Previous studies with lonidamine have shown that this agent produces a reduction in epididymal and testicular rABP with an increase in serum rABP (Lobl et al, 1981). These results were confirmed in the present study. Tolnidamine also produced profound alterations in rABP levels. The fall of epididymal rABP and rise of serum rABP is similar to what is observed with lonidamine; in contrast to the latter, however, tolnidamine produced an increase in testis rABP. This was evident whether results were expressed as tissue rABP concentration or as organ content. The reduction in epididymal rABP following treatment with either agent is most likely related to the inhibition of the passage of tubular fluid from the testis to the epididymis. Similar findings are observed with obstruction of the efferent ductules (Gunsalus et al, 1980), but obstruction seems unlikely in the

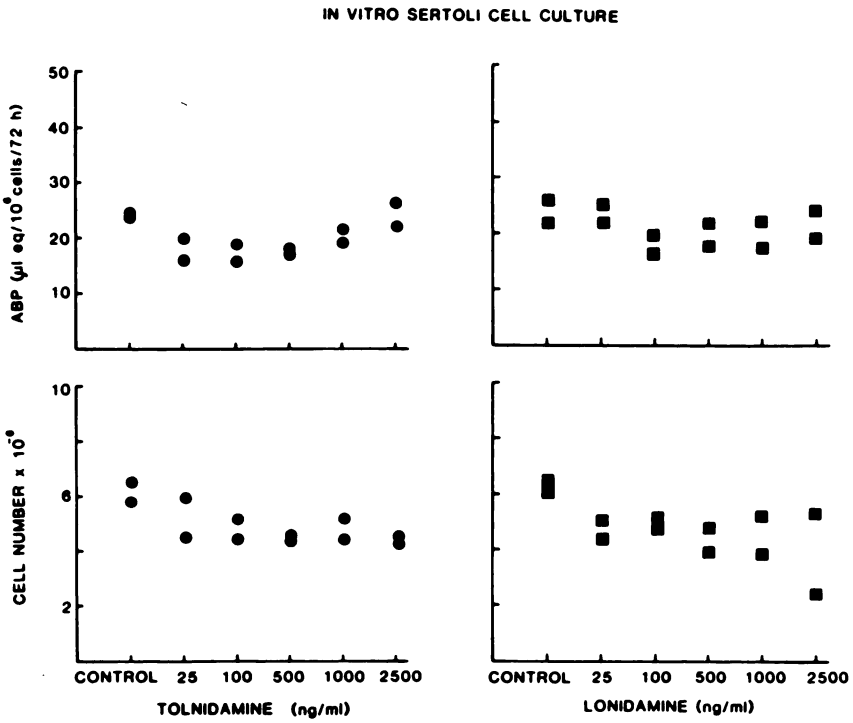


Fig. 5. Effect of tolnidamine (left) and lonidamine (right) on rABP secretion *in vitro*. Sertoli cell-enriched cultures were prepared from testes of 20-day-old rats as described in the text. Medium was changed on day 1 and 4 and cell numbers (lower panels) obtained on day 7. The results shown represent cumulative rABP secretion from days 4 to 7 (upper panels). Values for duplicate samples in a single experiment are shown.

present studies because testicular weights did not increase. An alternative explanation could be leakage of ABP from the epididymis into the blood. This usually occurs when the epididymis is regressing due to androgen deficiency (Becker et al, 1984), but this did not happen in our study, since testosterone levels remained normal.

The decrease in testicular rABP reported with lonidamine and the increase observed with tolnidamine suggest subtle differences of action of these drugs on the seminiferous tubule. The increase in testicular rABP with tolnidamine could be related to a reduction of rABP secretion into the seminiferous tubules without a proportional decline in synthesis. This could account for the rise in testicular, as well as the reduction in epididymal, rABP. The failure to observe significant changes in rABP concentration during incubation of cultured, hormonally-supplemented Sertoli cells from 20-day-old rats with increasing doses of tolnidamine or lonidamine implies the lack of a direct effect on rABP synthesis.

The rise in serum rABP could be related to the increased testicular rABP content. Tolnidamine could act directly on the basal aspect of the Sertoli cell, facilitating rABP release into the blood, as has been postulated to occur with progestins (Mather et al, 1983; Lobl et al, 1983). An alternative explanation is that tolnidamine might also disrupt the blood–testis barrier, leading to leakage of rABP into the serum, which is also believed to occur following lonidamine treatment. This latter drug crosses the blood–testis barrier and localizes in the seminiferous tubules and the tubular lumen (Hildebrandt-Stark et al, 1982). Further evidence for tight junction permeability following lonidamine administration is the penetration of lanthanum nitrate into the adluminal compartment (Buthala and Lobl, 1979). The observation of a rapid decline in testicular rABP associated with a rise in blood rABP after lonidamine is also consistent with disruption of the blood–testis barrier (Lobl et al, 1981). In this regard, lonidamine and tolnidamine appear to differ, since the latter agent produces a rise in both blood and testicular rABP.

We conclude that tolnidamine profoundly alters seminiferous tubular function, as evidenced by the marked alterations in testicular weights, as well as rABP concentrations in serum, testis, and epididymides. In contrast, Leydig cell function was spared, since testosterone levels were unchanged and only minimal alterations were observed in LH. FSH remained normal, implying that the factors that control rABP may be different from those that influence inhibin.

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