TOXICOLOGY AND APPLIED PHARMACOLOGY 81, 545–550 (1985)

## SHORT COMMUNICATION

# Comparative Effects of Diazepam and Midazolam on Paraoxon Toxicity in Rats[1,2]

Comparative Effects of Diazepam and Midazolam on Paraoxon Toxicity in Rats. KRUTAK-KROL, H., AND DOMINO, E. F. (1985). *Toxicol. Appl. Pharmacol.* **81**, 545–550. The present study was undertaken to compare the effectiveness of a new water-soluble benzodiazepine, midazolam, to diazepam, both administered im for protection against diethyl-*p*-nitrophenyl phosphate (paraoxon) toxicity. Adult male Sprague–Dawley rats were pretreated with midazolam or diazepam (0.32–32.0 mg/kg) alone or in combination with atropine (10.0 mg/kg). Twenty minutes later 2× LD50 of paraoxon was injected sc and the incidence of seizures and death were recorded for 24 hr. In another series of experiments, the LD50 of paraoxon was evaluated in the rats pretreated im with atropine (10.0 mg/kg) and midazolam or diazepam (10.0 mg/kg). Pretreatment with atropine alone did not prevent paraoxon-induced seizures but did reduce mortality. Both benzodiazepines were very effective alone or when combined with atropine in reducing the incidence of paraoxon-induced seizures. When given alone, neither benzodiazepine protected against paraoxon-induced mortality. However, when combined with atropine both benzodiazepines dramatically decreased the lethality of 2× LD50 of paraoxon. In equal doses given im, midazolam proved to be more potent than diazepam. 

It is generally accepted that inhibition of acetylcholinesterase (AChE) results in an accumulation of acetylcholine (ACh) which may be responsible for the acute toxic effects of organophosphorous (OP) compounds. Although atropine has long been used in the treatment of poisoning by acetylcholinesterase inhibitors (AChEI), including OP agents, it does not significantly reduce convulsions nor does it drastically alter their LD50 values. Inasmuch as antimuscarinic and ganglionic blocking agents alone or together do not provide sufficient antidotal activity against OP AChEIs, it follows that ACh may not be the only transmitter involved in their CNS actions. OP-induced convulsions may be related to reduced effectiveness of γ-aminobutyric acid (GABA) in the brain (Kar and Matin, 1972; Matin and Kar, 1973; Lundy *et al.*, 1978). Further studies on the detailed mechanisms involving GABA still need to be done.

Recent *in vitro* and *in vivo* studies (Haefely *et al.*, 1981; Meldrum and Braestrup, 1983) have shown that benzodiazepines enhance the postsynaptic inhibitory actions of GABA (both exogenous and synaptically released). Indeed, benzodiazepines are the most potent of the clinically available anticonvulsants, particulary when seizures are chemically induced by a large variety of substances. At least five reports have suggested that diazepam is potentially useful as an antidote against poisoning by AChEI (Lipp, 1972, 1973; Rump *et al.*, 1973; Johnson and Lowndes, 1974; Johnson and Wilcox, 1975). However, significant disadvantages are associated with the im administration of diazepam. The im use of this compound has almost disappeared from clinical practice (Reves *et al.*, 1984) because the drug is poorly absorbed, the injection is painful, and there is considerable local irritation. Therefore, evaluation of the anticonvulsant activity of novel, water soluble benzodiazepines such as midazolam is necessary. In general, midazo-

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lam, which shares the common pharmacodynamic properties and mechanisms of action with all benzodiazepines, has a shorter duration of action than does diazepam but is more potent. With regard to central nervous system GABA receptor binding, midazolam has a fivefold greater affinity than does diazepam (Mohler and Okada, 1977). Midazolam appears to be an ideal compound for im injection. After adminstration of the water-soluble preparation, the midazolam ring closes, causing the drug to become highly lipid soluble and to rapidly cross the blood brain barrier. The present study was initiated to determine if midazolam had any advantages over diazepam in the treatment of paraoxon toxicity in an animal model.

## METHODS

A total of 550 adult male Sprague–Dawley (Harlan Inc., Indianapolis, Ind.) rats, 250 to 400 g, were used in these experiments. They were maintained on a Purina rodent chow diet and were allowed free access to feed and water except during the time they were removed from their cages for testing. In general, there were five animals per cage. Subsequently, at the time of the experiment each animal was placed in its own cage. All experiments were performed between 9:00 to 11:00 AM since it has been previously shown that maximal sensitivity to seizures occurs during this period (Davis and Webb, 1963). All tested compounds were administered in a concentration that would keep the volume of the injection solution constant (1 ml/kg). Atropine and midazolam were dissolved in 0.9% NaCl immediately before injections. Drug doses were calculated as free base. Sources of the agents used were: atropine sulfate (Sigma Chemical Co., St. Louis, Mo. 63178), diazepam commercial solution (40% propylene glycol + 10% ethyl alcohol in water) and midazolam maleate (Hoffman–LaRoche Inc., Nutley, N.J. 07110), and paraoxon (Sigma).

*Study A.* Gross behavioral changes and toxic symptoms induced by paraoxon were evaluated. Animals were injected sc with paraoxon in a dose range of 0.032–1.0 mg/kg. In these and subsequent experiments the rats were examined visually for toxic symptoms such as initial changes in activity (calmness, hyperexcitability, sleep, stupor), salivation, lacrimation, piloerection, eyeball protrusion (exophthalmos), and evidence of respiratory distress. Ataxia, sensory evoked hyperactivity, tremor, jerks, and generalized convulsions were recorded. The type of seizure was analyzed as tonic (flexor and/or extensor) or clonic: mild, moderate (convulsions up to 5 sec), and severe (convulsed repeatedly for more than 5 sec). Recovery or death within 24 hr was evaluated. The mean convulsant dose 50% (CD50), the mean lethal dose 50% (LD50), and the 95% confidence limits were calculated by the method of Litchfield and Wilcoxon with an IMB PC computer (Tallarida and Murray, 1981).

*Study B.* Two series of experiments were undertaken. (1) Varying doses of atropine, diazepam, and midazolam alone or in combination were evaluated against 2× LD50 of paraoxon sc. Groups of eight rats at a minimum of five logarithmic doses of diazepam or midazolam (0.32–32.0 mg/kg) and atropine (1.0–100.0 mg/kg) were used. Both compounds were given im since this is the most practical and rapid route of antidote administration. Pretreatment time (20 min) prior to paraoxon was based on data concerning absorption and peak plasma concentrations of midazolam following its im administration to humans. The control group of animals were pretreated with 0.9% NaCl (saline) or 40% propylene glycol + 10% ethyl alcohol in water.

(2). The LD50s of sc paraoxon were evaluated in rats pretreated with atropine (10.0 mg/kg im), midazolam (10.0 mg/kg, im), and/or diazepam (10.0 mg/kg, im). The pretreatment time and route of drug injections were as above.

## RESULTS

### *Study A*

The sc administration of low doses (0.032–0.10 mg/kg) of paraoxon to rats induced slight gross behavioral changes like grooming, chewing, sniffing, and piloerection. A dose of 0.032 mg/kg caused (within 3–5 min) signs of cholinergic poisoning such as lacrimation, salivation, exophthalmos, fasciculations, tremor, and respiratory distress (bradypnea and dyspnea). Thirty percent of the animals developed moderate clonic seizures with a mild tonic component. Sixteen percent of the rats given a dose of 0.32 mg/kg died within 15–20 min. Adminstration of a larger dose (1.0 mg/kg) produced immediate toxic signs: tremor, jerks, and severe respiratory depression. All of these animals developed clonic seizures lasting 2–4 min until they died. Mortality in this group of rats was 100%.

The CD50 of paraoxon was determined to be 0.35 mg/kg with 95% confidence limits of 0.25–0.48. The LD50 calculated from the data was 0.38 mg/kg with 95% confidence limits of 0.27–0.52.

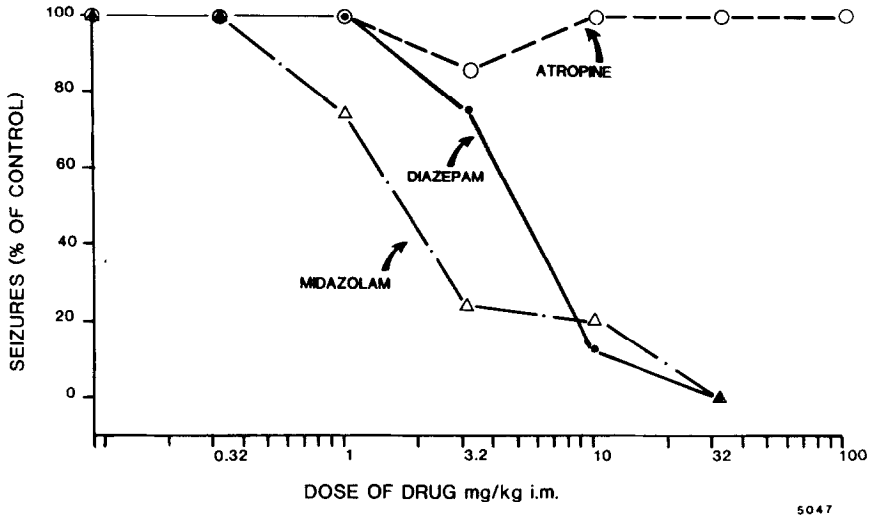


FIG. 1. Comparative effects of atropine, diazepam, and midazolam alone on paraoxon-induced seizures. The rats were pretreated with single, varying doses of atropine, diazepam, or midazolam given im. Twenty minutes later the animals were challenged with 2× LD50 (0.76 mg/kg) of paraoxon sc.

*Study B*

*1. Effect of atropine alone on paraoxon toxicity.* These results are summarized in Figs. 1 and 2. Rats (eight per group) were pretreated with im atropine in single doses (1.0, 3.2, 10.0, 32.0, and 100.0 mg/kg) 20 min prior to the sc administration of paraoxon (0.76 mg/kg = 2× LD50). The best protective effect against death was achieved by a 10.0 mg/kg dose of atropine (Fig. 1). Therefore, this dose was selected for subsequent experiments. Larger doses of atropine (32.0–100.0 mg/kg) increased mortality slightly compared to the 10.0 mg/kg dose. Pretreatment with atropine did not prevent paraoxon-induced seizures. However, it decreased salivation, lacrimation, and respiratory distress.

*2. Effect of midazolam alone.* In these series of experiments, rats (eight per group) were injected with midazolam im in single doses (0.32, 1.0, 3.20, 10.0, and 32.0, mg/kg). Low doses of midazolam produced no obvious effect.

With larger doses (3.2–10.0 mg/kg) rapid absorption of midazolam from the im injection was apparent after 2–3 min (i.e., rats became calm). By 5 min, all of the animals showed skeletal muscle relaxation and some developed ataxia but none fell asleep. A dose of 32.0 mg/kg of midazolam produced an anesthesia like state.

*3. Effect of midazolam pretreatment on paraoxon toxicity.* The incidence of paraoxon-induced convulsions was significantly reduced by prior adminstration of midazolam (Fig. 1). Doses of 3.2–10.0 mg/kg produced 75–80% protection against seizures produced by 2× LD50 of paraoxon. However, midazolam alone only slightly prevented death (Fig. 2). Pretreatment with both atropine (10.0 mg/kg) and midazolam (0.32–32.0 mg/kg) produced 100% protection against death. The combination of atropine (10.0 mg/kg) and midazolam (10.0 mg/kg) increased the LD50 of paraoxon to 4.12 (2.50–6.81 mg/kg, see Table 1). This pretreatment regime provided a protective ratio of 11.0.

*4. Effects of diazepam alone.* In another series of experiments, the effect of diazepam in single doses im was tested. Low doses (0.32–3.2 mg/kg) produced no obvious effect. With larger doses (10.0 mg/kg) the animal showed skeletal muscle relaxation and some developed ataxia. A dose of 32.0 mg/kg of diazepam induced an anesthesia like state. A delay of 10–

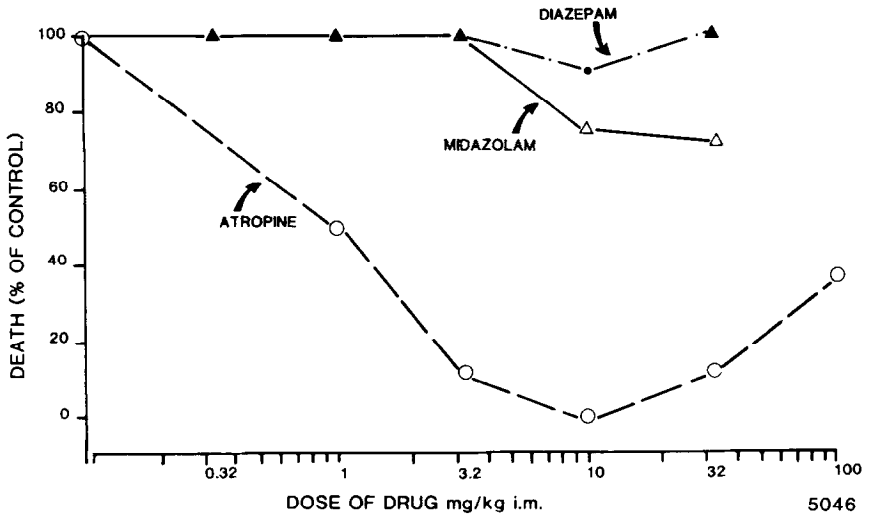


FIG. 2. Comparative effects of atropine, diazepam, and midazolam alone on paraoxon-induced death. The experimental procedure was as described in Fig. 1.

15 min from injection to occurrence of the first signs was seen.

*5. Effects of diazepam pretreatment on paraoxon toxicity.* In diazepam-pretreated animals there was a dose-dependent reduction in paraoxon-induced convulsions (Fig. 1). Small doses of diazepam (0.32–1.0 mg/kg) had no anticonvulsant activity. A dose of 32.0 mg/kg reduced convulsions by 25%. Doses of diazepam from 10.0 to 32.0 mg/kg provided 71.8–100% protection against seizures. However, diazepam alone, even in a very large dose (32.0 mg/kg), did not prevent paraoxon-induced respiratory distress and death (Fig. 2). Pretreatment with both atropine (10.0 mg/kg) and diazepam (10.0 mg/kg) provided 100% protection against death.

The combination of atropine (10.0 mg/kg) and diazepam (10.0 mg/kg) increased the LD50 of paraoxon to 2.72 (1.73–4.28) mg/kg (Table 1). This pretreatment regime provided a protective ratio of 7.16.

## DISCUSSION

The manifestations of OP AChEI toxicity include hypersecretion, motor hyperactivity, fasciculations, convulsions, respiratory distress, and death. The cause of death has been attributed to both central and peripheral respiratory depression. Atropine showed a beneficial effect on impaired respiration caused by paraoxon and increased the LD50 by a factor of 3.1. The present studies confirm the finding that atropine is effective as an antidote

TABLE 1

PROTECTIVE EFFECT OF ATROPINE, MIDAZOLAM, OR DIAZEPAM AGAINST PARAOXON[a] MORTALITY

| Compound (mg/kg)[b] | $n$[c] | LD50 (95% confidence limits) (mg/kg) |
|---|---|---|
| No pretreatment | 73 | 0.38 (0.27–0.52) |
| 0.9% NaCl | 25 | 0.33 (0.21–0.52) |
| Atropine 10.0 mg/kg | 32 | 1.18 (0.97–1.42) |
| Diazepam 10.0 mg/kg | 43 | 0.35 (0.13–0.92) |
| Midazolam 10.0 mg/kg | 61 | 0.57 (0.29–1.12) |
| Diazepam 10 mg/kg + atropine 10.0 mg/kg | 83 | 2.72 (1.73–4.28) |
| Midazolam 10.0 mg/kg + atropine 10.0 mg/kg | 72 | 4.17 (2.50–6.81) |

[a] Doses of paraoxon used were: 0.76, 1.0, 1.52, 2.0, 3.0, 4.0, 6.0 mg/kg.

[b] Atropine and midazolam or diazepam were injected im 20 min prior to the sc administration of paraoxon.

[c] Number of animals used.

to OP-induced respiratory failure (Johnson and Wilcox, 1975; Lipp, 1976). Atropine appears to have at least three mechanisms of action in reducing respiratory depression: (1) it blocks the interaction between ACh and cholinergic receptors in the central respiratory center, (2) it reduces tracheobronchial secretion, and (3) it increases the heart rate which possibly enhances blood flow to the brainstem respiratory centers, thus reducing the anoxia which results in activation of respiratory neurons (Lipp, 1976). However, atropine, when injected alone, does not antagonize seizures induced by paraoxon. We hypothesized that if the convulsions produced by paraoxon (and probably other OP agents) were solely a result of activation of the cholinergic system, high doses of atropine would have abolished the convulsions, since atropine pretreatment prevents paraoxon-induced increases in brain ACh amounts (Wecker *et al.*, 1977). Large doses (32.0–100.0 mg/kg) of atropine did not antagonize paraoxon-induced seizures and were less effective in protecting against death than smaller doses (10.0 mg/kg) of atropine.

Since atropine does not provide complete protection against OP poisoning, other forms of therapy have been investigated. Benzodiazepines have been reported to provide both protective and antidotal activity against poisoning by OP AChEI (Lipp, 1972, 1973, 1974; Rump *et al.*, 1973; Johnson and Lowndes, 1974; Lundy *et al.*, 1978; Jones *et al.*, 1984). In our experiments, midazolam im had no effect on paraoxon-induced stimulation of mucous secretions and impaired respiration, but was very effective in suppressing seizures. Diazepam showed a similar effect, but it was less potent.

Davies and Polc (1978) showed that iontophoretically administered midazolam antagonized the excitatory effects of ACh on single neurons, but when injected iv, midazolam did not show anticholinergic properties. Therefore, it is very unlikely that the potent anticonvulsant effects of midazolam could be due to its anticholinergic activity. The mechanism of action of midazolam appears to be potentiation of the inhibitory effect of GABA (Cheng and Brunner, 1981). Since midazolam is effective in suppressing paraoxon-induced seizures, one can conclude that enhancement of GABA mechanisms reduces paraoxon-induced seizures. Perhaps paraoxon induces seizures by a non-cholinergic mechanism related to inhibition of GABA. Of course, the other possibility remains that paraoxon-induced convulsions are mediated through the release of an excitatory transmitter such as glutamate, etc, in the brain which is affected by benzodiazepines directly or indirectly.

In the present study, both benzodiazepines were very effective when combined with atropine in reducing the incidence of paraoxon-induced seizures and dramatically reducing the lethality of 2× LD50 of paraoxon. However, midazolam was more potent on a milligram per kilogram basis.

In atropine/midazolam-pretreated animals, the LD50 of paraoxon was increased by a factor of 11.0. Diazepam, when combined with atropine, increased the LD50 of paraoxon by 7.16. When given im to humans, midazolam is better absorbed than is diazepam. Midazolam, contrary to the present commercial diazepam solution, is compatible when mixed with other agents in the same syringe. Thus, midazolam would be preferred in treating paraoxon poisoning, assuming that the rat data are applicable to humans.

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