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# Effects of Midazolam on T-Cell Immunosuppressive Response to Surgical Stress in Mice

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FREIRE-GARABAL, M., A. BELMONTE, J. L. BALBOA AND M. J. NÚÑEZ. *Effects of midazolam on T-cell immunosuppressive response to surgical stress in mice.* PHARMACOL BIOCHEM BEHAV **43**(1) 85–89, 1992.—Mice submitted to surgical stress induced by laparotomy and treated with chronic midazolam (1 mg/kg) showed a reduction in stress-induced suppression of thymus and spleen cellularity and in peripheral lymphocyte population. The blastogenic response of spleen lymphoid cells was also assessed and midazolam was found to partially attenuate the suppressive effect of surgery.

Stress Immunity T Lymphocytes Blastogenic response Midazolam Anxiolytics ACTH

RESEARCH has proved that surgical stress may induce adverse effects on host defense mechanisms, particularly cell-mediated immune responses (18,20,22,31–33,41,45,47). It was found that stress could suppress T-cell populations, the natural killer cytotoxicity, and the proliferation of spleen cells induced by concanavalin A (Con A) (31). Long-term studies also demonstrated that the stress-mediated immunosuppression was reversible as the responses to Con A were restored with time (47).

Stress-induced modulation of immunity has a neuroendocrine basis and requires the presence of pituitary, as demonstrated in hypophysectomized rats (19). At least one of the neuroendocrine responses to stress, for example, the rise in plasma corticosterone concentrations via corticotropin (ACTH) secretion, has an easily demonstrable destructive effect on specific cells and tissues that are required for optimum immunological defense (3,16,24,25,28,34,42).

It has been reported that anxiolytics can attenuate the response of ACTH and corticosterone to stress (21,30), but there is little data on its effect on the number and function of immune cells. This present article studies the quantitative effects of midazolam, a benzodiazepine agonist, on T-cell populations, the blastogenic response of spleen cells to Con A, and plasmatic ACTH concentrations after surgical stress in mice.

## METHOD

### *Mice*

Female mice (7–10 weeks old at the beginning of the experiment) of the BALB/c strain (Interfauna Ibérica S.A., Barcelona, Spain) were used. They were housed, 7 days before experiments, four per cage in an aseptic chamber kept between 21 and 22°C and maintained on an alternating 12 L : 12 D cycle. Sterilyzed food (Panlab Diet A.03) and water were given ad lib.

### *Procedure*

All experimental mice were treated with vehicle or midazolam (1 mg/kg) 1 h before and 12, 24, and 48 h after surgery. Animals were killed to obtain samples, 18 per group (vehicle and midazolam), at time 24, 48, and 72 h. Three lots of six animals per group were used to determine, respectively, cell populations, blastogenic response of spleen cells, and plasmatic levels of ACTH.

### *Induction of Surgical Stress*

Mice were subjected to a medial laparotomy under ether anesthesia in aseptic conditions. The abdominal wall was cut and the intestines were agitated. Surgery was always per-

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formed at 5:00 p.m. Unstimulated controls were exposed only to the normal activity of the animal room.

*Quantification of Thymic, Splenic, and Blood Cellularity*

To determine the number of cells in thymuses and spleens, mice were killed by cervical dislocation and organs were removed, weighed, and forced through a stainless steel mesh screen to form a single cell suspension. Cell counts were determined with a hemacytometer on appropriate dilutions of cell suspensions. Viability was estimated by exclusion of tripan blue. For peripheral blood counts, mice were bled from the retro-orbital venous plexus. Cell numbers were determined with a Coulter counter. Slides of peripheral blood were stained with Wright's stain and differential counts were performed on 200 cells/slide.

*T-Cell Proliferation Assay*

Spleens were removed aseptically and passed through a no. 60 wire mesh. The cells were rinsed from the wire mesh and centrifuged at 200 × *g* for 15 min. Erythrocytes were lysed with 0.83% $NH_4Cl$ buffered to pH 7.5 with Tris for 10 min and then washed in Hanks balanced salt solution. Cells were then resuspended into medium and cultured in round-bottomed microdilution plates. The culture medium was RPMI 1640 (GIBCO Laboratories, Gran Island, NY) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES buffer (10 mM), 2-mercaptoethanol (2-ME; $5 \times 10^{-2}$ M), and 10% fetal calf serum (GIBCO). In a 200-μl volume, there were $10^5$ cells with concentrations of Con A (Pharmacia Diagnostics, Piscataway, NJ) at 10 μg/ml (46). Plates were incubated at 37°C in a humidified 5% $CO_2$ incubator for 3 days. During the final 18 h of incubation, 10 μl medium containing 0.5 μCi [$^3$H]thymidine ([$^3$H]TdR; 6.7 Ci/mol; New England Nuclear Corp., Boston, MA) was added. Cells were collected on glass filter paper. Filters were air dried, placed on scintillation vials containing 5 ml 3a 7oB scintillation fluid mixture, and the cell-associated [$^3$H]radioactivity determined on a B scintillation counter. Each sample was counted for 5 min, and net counts per minute of six spleens per datum were recorded and expressed as the mean ± SD (39,47).

*Plasmatic ACTH Determinations*

Mice used in this experiment were killed by decapitation and blood from the trunk was collected by gravity in heparinized beakers containing 1,000 KIU of trasylol (Sigma Chemical Co., St. Louis, MO). The blood was centrifuged at 3,000 × *g* for 10 min at 4°C and plasma was stored at −70°C until assayed. ACTH was measured using a radioimmunoassay kit (Immuno Nuclear Corp, Madrid, Spain). Human ACTH was used as standard. The assay was performed in 10 × 75 polystyrene tubes using an overnight incubation at 4°C. Assay sensitivity was approximately 10 pg/ml. The intraassay coefficient of variation was 2.5% at 380 pg/ml and the interassay coefficient of variation was < 5%.

*Drug Treatments*

The drug tested was midazolam (Roche S.A., Madrid, Spain), which was intraperitoneally injected in a dose of 1 mg/kg (17). Control stressed mice were intraperitoneally injected with vehicle as placebo. Volume of all injections was 1 ml/kg.

*Statistical Analysis*

Statistical analysis was performed using Student's *t*-test, $\chi^2$ analysis, or one-way analysis of variance (ANOVA) with grouping of means by Fisher's least-significant difference method (37).

## RESULTS

Table 1 shows the results of differential cell counts performed on thymuses, spleens, and peripheral blood. Mice killed 24 h after surgery showed a sharp fall in thymus cellularity: 95% for vehicle and 40% for the midazolam-treated group. Both experimental groups recovered cellularity 48 h after laparotomy, but values were higher in midazolam-treated mice. Stressed mice showed an involution of thymus weight similar to thymic cellularity (Table 2) that is also attenuated by midazolam. Changes in spleen cells and peripheral blood lymphocytes were similar but not as marked.

Spleen cells obtained 24 and 48 h after surgery were cultured and assayed for blastogenic response to Con A (Fig. 1). The results indicate that stress induces a decrease in blastogenic response to mitogen Con A in both experimental groups compared with unoperated controls. Nevertheless, the decrease was more than 77% for vehicle and less than 42% for midazolam-treated mice (differences: $p < 0.01$).

Results obtained in plasmatic ACTH concentrations are presented in Table 3. These data show that midazolam sig-

TABLE 1

CELL NUMBER/ORGAN IN MICE SUBMITTED TO SURGICAL STRESS AND TREATED WITH PLACEBO OR MIDAZOLAM

| Hours After Surgery | Placebo | Midazolam | Placebo | Midazolam | Placebo | Midazolam |
|---|---|---|---|---|---|---|
| 24 | 0.42 ± 0.28 | 8.25 ± 0.34* | 4.37 ± 0.45 | 7.45 ± 0.56* | 2.89 ± 0.45 | 8.67 ± 0.61* |
| 48 | 2.83 ± 0.65 | 9.37 ± 0.44* | 3.61 ± 0.49 | 8.57 ± 0.34* | 4.10 ± 0.49 | 8.98 ± 0.56* |
| 72 | 3.11 ± 0.41 | 10.12 ± 0.34* | 6.58 ± 0.41 | 9.34 ± 0.29* | 2.41 ± 0.41 | 9.34 ± 0.51* |

Unoperated controls: thymus × $10^7$, 12.32 ± 0.39; spleen × $10^7$, 9.44 ± 0.42; peripheral blood lymphocytes × $10^3/mm^3$, 9.66 ± 0.38.

Organ and peripheral blood counts of stressed mice were determined at time 0, 24, 48, and 72 h of experiments in both placebo- and midazolam-treated groups. Six mice were used for each condition. Values represent mean ± 1 SD. Values for each organ were analyzed by one-way ANOVA and grouped by Fisher's least-significant difference method.

*Values significantly greater ($p < 0.01$) than placebo values.

TABLE 2

EFFECTS OF SURGICAL STRESS AND MIDAZOLAM IN THYMUS WEIGHT

| Hours After Surgery | Placebo | Midazolam |
|---|---|---|
| 24 | 0.45 ± 0.10 | 1.98 ± 0.21* |
| 48 | 1.41 ± 0.37 | 2.20 ± 0.34* |
| 72 | 1.93 ± 0.24 | 2.36 ± 0.28* |

Unoperated controls: weight (mg thymus/g body weight), 2.55 ± 0.13.

Values represent the mean ± 1 SD, which were analyzed by ANOVA and grouped by Fisher's least-significant difference method. Thymus involution in mice subjected to a surgical stressor is attenuated by midazolam.

*Values significantly greater ($p < 0.01$) than placebo values.

TABLE 3

THE EFFECTS OF SURGICAL STRESS AND MIDAZOLAM ON PLASMATIC ACTH LEVELS

| Hours After Surgery | Placebo | Midazolam |
|---|---|---|
| 24 | 520.31 ± 15.8 | 429.45 ± 25.7* |
| 48 | 487.24 ± 30.2 | 379.83 ± 32.0* |
| 72 | 490.73 ± 28.9 | 364.70 ± 10.3* |

Unoperated controls: ACTH (pg/ml), 312.06 ± 21.7.

Values represent the mean ± 1 SD, which were analyzed by ANOVA and grouped by Fisher's least-significant difference method. Rises in plasma ACTH induced by surgical stress were significantly attenuated by midazolam.

*Values significantly greater ($p < 0.01$) than placebo values.

nificantly attenuates the rise in plasma ACTH induced by stress.

## DISCUSSION

Surgical stress caused a decrease in all lymphocyte populations assayed. Thymic and spleen cellularity, as well as peripheral blood lymphocytes, fell sharply after laparotomy. We also observed an inhibitory effect stress on the blastogenic response of spleen cells induced by Con A.

While the immunosuppressive properties of short-term exposures to various stressors, such as surgery, have been well established (20,29,32,34,41,43), the effects of long-term stress on the immune response are less clear. Monjan and Collector (27) observed that an auditory stressor first depressed immune responsiveness but later enhanced it.

A molecular basis for bidirectional communication between the immune and neuroendocrine systems has been described (1,5-11,13,36,38). First, cells of the immune system can synthesize biologically active neuroendocrine peptide hormones. Second, immune cells also possess receptors for many of these peptides. Third, these same neuroendocrine hormones can influence the immune function. Fourth, lymphokines can influence neuroendocrine tissues. The nervous system's recognition of stimuli can be converted into chemical signals that can be relayed to immune cells, resulting in physiological changes (44).

Stress-induced immunosuppression is considered, among other factors (4,14,21,23,26,35,40), as a consequence of increased plasma corticosterone concentrations, a key factor in causing impairment of the rodent immunological apparatus (2,12,15,19,28,34,42). Nevertheless, Monjan and Collector

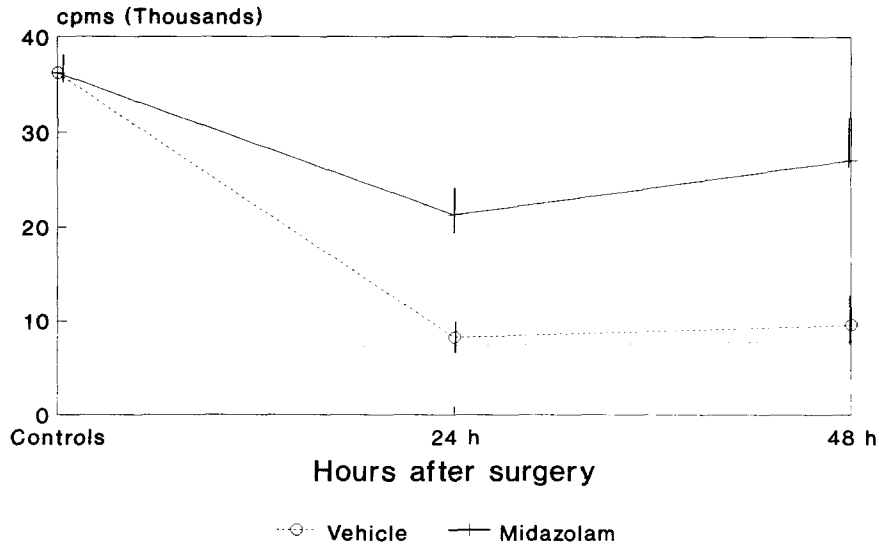


FIG. 1. Response of spleen cells, under in vitro conditions, to mitogen Con A 24 and 48 h after surgical stress (laparotomy) in mice treated with vehicle and midazolam. Each point represents the mean ± 1 SD of six spleens per datum. For control animals, the mean number of counts per minute were 36,217 for Con A.

observed levels of cortisol in plasma appeared to be temporally related to immunological hyporeactivity but not to hyperreactivity. Yang and Ma (47) observed a restoration but no enhancement of the immune response with time.

Midazolam is a benzodiazepine agonist currently used in anesthesia. Besides its anxiolytic effects, it is a sedative compound that deepens anesthesia and, at least in humans, potentiates perioperative amnesia. Previous findings in rodents show that benzodiazepines inhibit the rise of ACTH and corticosterone concentrations during stress (3,25), as was also observed for midazolam in this experiment.

Adverse effects of surgery were lower in mice injected with midazolam. Thymus weight and cellularity, as well as spleen cellularity, were greater than those of mice injected with placebo although values remained below those of the controls. Similar effects were observed in peripheral lymphocyte populations. In vitro studies also show an inhibitory effect of surgical stress on blastogenic response of spleen cells that is attenuated by administration of midazolam.

Our results are in good agreement with those of Okimura and Nagata (29) who observed that diazepam promoted the antibody response through stimulating helper T-cell functions in restraint-stressed mice.

Plasmatic ACTH determinations suggest an interaction between corticoid modulation by midazolam and the T-cell immune response. Nevertheless, in previous studies not reported here, we observed that adrenalectomized mice showed a lower pattern of immunosuppression in response to stress. So, this leads us to believe that other neuropeptides and neurotransmitters may be involved in the immunosuppressive response to stress (4,13,21,23,26,35,40) and in the action of anxiolytics.

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