Effects of diazepam and flumazenil on forebrain ischaemia in a rat model of benzodiazepine tolerance

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Editor’s key points

• Benzodiazepine treatment is neuroprotective after cerebral ischaemia.
• Benzodiazepine tolerance reduces this neuroprotection.
• The benzodiazepine antagonist flumazenil restored neuroprotection by diazepam in benzodiazepine-tolerant animals after cerebral ischaemia in rats.
• These findings are clinically relevant.

Background. Post-ischaemic benzodiazepine administration is neuroprotective, but chronic administration of benzodiazepines can induce tolerance, such that the neuroprotective effect may be reduced. This study investigated whether benzodiazepine tolerance can worsen ischaemic injury and whether neuroprotection by post-ischaemic benzodiazepine administration is affected by benzodiazepine tolerance. We also investigated whether antagonism of benzodiazepine receptors by flumazenil was able to restore neuroprotection during benzodiazepine tolerance.

Methods. Experiments were performed in both benzodiazepine-tolerant and naive rats. Benzodiazepine tolerance was induced by 4 weeks administration of flurazepam. Bilateral carotid artery occlusion (BCAO) was performed to cause cerebral ischaemia. Four experiments were performed: (1) BCAO with no further interventions; (2) BCAO followed by administration of diazepam; (3) administration of flumazenil before BAO; and (4) administration of flumazenil before and diazepam after BCAO. Neurological and histological assessment was performed 5 days after BCAO.

Results. Benzodiazepine tolerance did not affect neuronal injury in the CA1 and CA3 regions and dentate gyrus of the hippocampus after severe ischaemic insult, but did worsen neuronal damage when mild ischaemia was applied (P<0.05). Neuroprotective efficacy of post-ischaemic diazepam was not observed under conditions of benzodiazepine tolerance. Flumazenil treatment before BCAO reduced ischaemic neuronal damage exacerbated by benzodiazepine tolerance (P<0.05), and restored neuroprotection by post-ischaemic diazepam (P<0.05), the effect of which was reduced by benzodiazepine tolerance (P<0.05). However, pre-ischaemic flumazenil treatment in naive animals reduced neuroprotection provided by post-ischaemic diazepam (P<0.01–0.05).

Conclusions. Benzodiazepine tolerance can worsen ischaemic neuronal injury and abolish the neuroprotection provided by post-ischaemic diazepam. Pre-treatment with flumazenil treatment reversed benzodiazepine tolerance and restored neuroprotection by post-ischaemic diazepam. These findings may suggest that management of patient’s risk of developing cerebral ischaemia may need to take into account current use.

Keywords: brain ischaemia, cerebral ischaemia; cerebrovascular occlusion, bilateral carotid artery occlusion; drug tolerance, benzodiazepine tolerance; flumazenil; neuroprotective drug, diazepam

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Consumption of benzodiazepine has increased in modern society because of an increased number of patients with insomnia or anxiety.1,2 Chronic administration of benzodiazepines can induce tolerance against these conditions in clinical settings. In animal experiments, it has been reported that oral administration of flurazepam for 4 weeks induces tolerance.3–6 Benzodiazepine receptor down-regulation occurs during chronic treatment and this contributes to the tolerance,3–6 with a reported 20% decrease in the receptor number.3–7 Extracellular γ-aminobutyric acid (GABA) increases immediately after ischaemia as a self-defence mechanism. Therefore, external GABA stimulation by benzodiazepines during this period has no further neuronal protective effect because the inhibitory neurotransmission system is already saturated by internal GABA accumulation.8 However, subsequent benzodiazepine administration has a profound neuroprotective effect.9–14 The mechanisms contributing to benzodiazepine neuroprotection are unclear, but post-ischaemic benzodiazepine administration is clearly protective.
Therefore, the inhibition of benzodiazepine pharmacodynamics is thought to be related to the inhibition of this neuroprotective effect. In other words, the inhibition of GABA neurotransmission may be induced during benzodiazepine tolerance, which might worsen ischaemic injury. Moreover, benzodiazepine tolerance may reduce the neuroprotective effect produced by benzodiazepine.

Based on this hypothesis, the following studies were conducted. First, we examined whether benzodiazepine tolerance worsens ischaemic injury and whether neuroprotection by post-ischaemic benzodiazepine administration is affected by benzodiazepine tolerance. This raised the question of whether benzodiazepine tolerance is reversible, and whether the benzodiazepine neuroprotective effect can be restored once benzodiazepine tolerance is induced. It has been reported that the antagonism of benzodiazepine receptors by flumazenil rapidly reverses benzodiazepine tolerance. Therefore, deterioration of neuronal injury during benzodiazepine tolerance may be improved by normalization of GABA neurotransmission with flumazenil treatment. The antagonism of benzodiazepine receptors by flumazenil may also restore the benzodiazepine neuroprotective effect during benzodiazepine tolerance. Therefore, the effect of flumazenil on forebrain ischaemia during benzodiazepine tolerance and that of flumazenil on neuroprotection with post-ischaemic diazepam administration during benzodiazepine tolerance were investigated in a rat model of benzodiazepine tolerance.

**Methods**

All experimental protocols were approved by the Animal Care and Use Committee of Nara Medical University.

**Inducing benzodiazepine tolerance**

Male Sprague–Dawley rats (Japan SLC, Shizuoka, Japan; weight 200–250 g) were used in the study. Rats were given flurazepam in a 0.02% saccharine solution as their only source for 4 weeks. The drug concentration was adjusted to provide up to 100 mg kg⁻¹ daily for the first week and 150 mg kg⁻¹ day⁻¹ for the next 3 weeks. This treatment leads to tolerance to locomotor impairment upon injection of a large test dose of benzodiazepine. Control rats received saccharine solution only. For inclusion in the study, rats had to have consumed a minimum average dose of 100 mg kg⁻¹ flurazepam daily. Residual flurazepam and metabolites decline in the hippocampus after the first 24 h after drug removal, with corresponding plasma half-lives of <2 h.

**Inducing bilateral carotid artery occlusion**

Bilateral carotid artery occlusion (BCAO) was induced 24 h after cessation of the 4 week treatment with flurazepam. Anaesthesia was induced with 5% isoflurane and the trachea was intubated. The isoflurane concentration was then reduced to 1.5–2%. Adequate anaesthesia was confirmed by the lack of spontaneous movement during surgical procedures. Ventilation was adjusted to achieve PaCO₂ of 35–40 torr and PaO₂ of >100 torr. Body temperature was maintained at 37.5 (0.5)°C by surface heating or cooling. The mean arterial pressure (MAP) and heart rate were monitored throughout the experiment. The anaesthetic concentration was maintained at 1.5% isoflurane, heparin (100 units kg⁻¹) was administered i.v., and then hypotension was induced by withdrawal of blood from the superior vena cava catheter into a pre-warmed syringe. Once the MAP decreased to 35 mm Hg, both carotid arteries were occluded with vascular clamps for 6 or 8 min. During the occlusion period, MAP was maintained at 35 mm Hg by withdrawal or reinfusion of blood. After the period of ischaemia, reperfusion of the brain was established by removal of the vascular clamps and reinfusion of the withdrawn blood. The vascular catheters were removed and the wounds were closed. All wounds were infiltrated with 0.25% bupivacaine. The animal was transferred to a pre-warmed oxygen-rich humidified recovery chamber maintained at a temperature of 22°C.

See Supplementary material for further details.

**Neurological assessment**

At 5 days post-BCAO, neurological assessment of motor activity was performed by an investigator blinded to the group assignment using the modified method of Sano and colleagues. After assessment, the animal was reanaesthetized using 2.5–3.0% isoflurane through a mask and sodium thiopental (2–3 mg kg⁻¹ i.p.) was then injected and tissues were removed for histological assessment as described below. See Supplementary material for further details.

**Harvesting for histological assessment**

Paraffin-embedded coronal sections (5 µm) of the brain were prepared and stained with haematoxylin and eosin. An investigator blinded to the group assignment evaluated the CA1 and CA3 sectors and the dentate gyrus (DG) of the hippocampus in a coronal plane 3300 µm posterior to the bregma. These regions were determined on each slice by referring to the rat brain atlas of Palkovits and Brownstein. Viable and nonviable neurons were counted manually, and the percentage of nonviable neurons was calculated for quantification of neuronal damage in a 0.5 × 0.5 mm counting frame at a magnification of ×200. Neuronal damage was reported as the percentage of non-viable neurons.

See Supplementary material for further details.

**Effect of forebrain ischaemia on benzodiazepine-tolerant rats (Fig 1)**

BCAO (8 min in Experiment 1A and 6 min in Experiment 1B) was induced 24 h after flurazepam or control treatment. After recovery, rectal temperature was monitored without intervention. Five days after BCAO, animals underwent a neurological examination and were then killed for histological assessment. In another set of animals, normothermia was maintained for 3 h after recovery from BCAO to evaluate the effect of benzodiazepine tolerance on body temperature.
Effect of benzodiazepine tolerance on neuroprotection by post-ischaemic diazepam administration (Fig 1)

Based on the results of Experiment 1, 8 min BCAO was used in Experiment 2. BCAO was induced 24 h after cessation of flurazepam or control treatment. After recovery from BCAO, the rectal temperature was monitored for 4 h without intervention. Diazepam 10 mg kg\(^{-1}\) or vehicle (33% polyethylene glycol in saline) was given i.p. 1 and 2 h after reperfusion. Five days after BCAO, the animals underwent neurological examination and histological assessment.

Effect of flumazenil on forebrain ischaemia during benzodiazepine tolerance (Fig 1)

Based on the results of Experiment 1, 6 min BCAO was used in Experiment 3. Twelve hours after flurazepam or control treatment, 20 mg kg\(^{-1}\) flumazenil was given i.p. BCAO was performed 12 h after this. After recovery from BCAO, rectal temperature was monitored for 3 h without intervention. Five days after BCAO animals underwent neurological examination and histological assessment.

Effect of flumazenil on neuroprotection by post-ischaemic diazepam administration during benzodiazepine tolerance (Fig 1)

Based on the results of Experiments 1 and 3, 8 min BCAO was used in Experiment 4. Twelve hours after flurazepam or control treatment, 20 mg kg\(^{-1}\) of flumazenil was given i.p. BCAO was performed 12 h after this. After recovery from BCAO (8 min), rectal temperature was monitored for 4 h without intervention. Diazepam 10 mg kg\(^{-1}\) or vehicle (33% polyethylene glycol in saline) was given i.p. 1 and 2 h after reperfusion. Five days after BCAO, animals underwent neurological examination and histological assessment.

Statistical analysis

Physiological data, neurological assessment, and cell counts were compared among groups by unpaired t-test, analysis of variance (ANOVA), or repeated-measures ANOVA. If the ANOVA identified significant differences, an unpaired t-test with a Bonferroni correction was used for intergroup comparison. Mortality was compared by the \(\chi^2\) test or the Fisher exact test. All data except for mortality rate are shown as mean (SD). Statistical analysis was performed with Statview 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Neuroprotection by diazepam was not observed in our laboratory after BCAO when body temperature was maintained at 37.5°C. Therefore, post-ischaemic body temperature was monitored but not controlled. The rectal temperature of most animals tended to decline in the early period after ischaemia (Figs 2 and 3). However, recovery to normothermia seemed to be achieved earlier after a shorter period of ischaemia (6 min) compared with a longer period (8 min). Diazepam fostered the post-ischaemic body temperature decline. Several rats died during the 5 day recovery period (see Supplementary material for further details), including death during the early recovery period due to apparent upper airway obstruction or seizure. The causes of other deaths were not identified. Physiological values for \(P_{a\text{CO}_2}\)
and \( P_{O_2} \) were maintained around the target values described in the Methods section, and pre-ischaemic physiological variables including Hct, glucose, MAP, and heart rate were similar among the groups. Pre-ischaemic body weight ranged from 370 to 400 g and did not differ among the groups. Weight loss after the 5 day recovery period was not significant in all groups and rectal temperature before death was similar among the groups (data not shown).

Throughout Experiments 1–4, the neurological outcome showed no differences among the groups (see Supplementary material for further details).

**Experiment 1**

Rectal temperature after ischaemia showed similar changes in the benzodiazepine-tolerant and naive animals. The value in the naive animals 3 h after ischaemia was significantly higher than that in the benzodiazepine-tolerant animals, but both values were under normothermia (Fig. 2, Experiment 1A). The percentage of injured neurones in the CA1 and CA3 sectors and the DG after 8 min ischaemia did not differ between benzodiazepine-tolerant and control animals (Fig. 2). Rectal temperature after ischaemia was significantly higher in the benzodiazepine-tolerant animals compared
with the naive animals, and was over normothermia (Fig. 2, Experiment 1B). The percentage of injured neurones in the CA1 and CA3 sectors and the DG was significantly increased by induced benzodiazepine tolerance compared with controls after 6 min BCAO (Fig. 2). When normothermia was maintained after 6 and 8 min BCAO, there was no difference in the percentage of injured neurones between the two groups (data are not shown). Representative pictures of the hippocampus after 6 and 8 min of BCAO in the benzodiazepine-tolerant and naive animals are shown in the Supplementary material.

**Experiment 2**
Rectal temperature after ischaemia in diazepam-treated animals in the naive group tended to be lower than that in other animals (Fig. 3). Post-ischaemic diazepam significantly reduced the percentage of injured neurones after ischaemia in the absence of benzodiazepine tolerance. Similar to the results of Experiment 1, induced benzodiazepine tolerance did not significantly worsen ischaemic neuronal injury compared with without benzodiazepine tolerance. However, post-ischaemic diazepam failed to reduce the percentage of injured neurones after ischaemia when benzodiazepine tolerance was induced. In the CA3 sector and the DG, post-ischaemic diazepam had no significant effect on the percentage of injured neurones between tolerant and naive animals (Fig. 3).

**Experiment 3**
Rectal temperature after ischaemia in vehicle-treated animals in the benzodiazepine-tolerant group tended to be higher than that in other animals (Fig. 3). Pre-ischaemic flumazenil treatment reduced the percentage of injured neurones after ischaemia, even when benzodiazepine tolerance was induced, and the levels were similar to the percentages in animals without benzodiazepine tolerance or pre-ischaemic flumazenil treatment.

**Experiment 4**
Rectal temperature after ischaemia declined in all groups. As in Experiment 2, induced benzodiazepine tolerance did not reduce the percentage of injured neurones with post-ischaemic diazepam. However, post-ischaemic diazepam with pre-ischaemic flumazenil treatment significantly decreased the percentage of injured neurones, even when benzodiazepine tolerance was induced. The extent of this effect was almost equal to the neuroprotective effect of diazepam in the absence of benzodiazepine tolerance. In contrast, pre-ischaemic flumazenil treatment without benzodiazepine tolerance increased the percentage of injured neurones, even with post-ischaemic diazepam, and the level of injured neurones was similar to that with induced benzodiazepine tolerance (Fig. 3).

**Discussion**
We have shown that benzodiazepine tolerance can exacerbate ischaemic injury and reduce neuroprotection by diazepam in rats and this can be reversed by treatment with flumazenil. These findings suggest that benzodiazepine users may be susceptible to cerebral ischaemia, and may fail to benefit from post-ischaemic treatment with diazepam. Flumazenil may reverse these effects. Clinical use of flumazenil before transient global cerebral ischaemia, which is representative of cerebral ischaemia in cardiac arrest, may be limited. However, flumazenil may be considered in situations such as major cardiovascular or cerebrovascular surgeries.

Post-ischaemic administration of diazepam is neuroprotective against transient global cerebral ischaemia.9–14 Substantial neuroprotection of striatal and pyramidal neurones in the CA1 area of the hippocampus was reported after diazepam administration into rats after transient global ischaemia.9 Neuroprotection in CA1 pyramidal cells of the hippocampus was also found after diazepam administration into gerbils after transient global ischaemia.10 Diazepam is a specific agonist of the benzodiazepine receptor that increases inhibition by acting on GABA inhibitory effects that may protect neurones during ischaemia.20 Therefore, enhancement of GABA neurotransmission by diazepam after an ischaemic event may offset neuronal excitability and prevent neuronal death.9, 10 However, diazepam is also known to induce hypothermia.11–13 21 22 and post-ischaemic mild hypothermia provides neuroprotection of the hippocampus in rodents.23 Therefore, induced hypothermia may be partly responsible for the neuroprotection exerted by diazepam.12 13 21 22 Indeed, we did not observe a neurotoxic effect of benzodiazepine tolerance or neuroprotective efficacy of post-ischaemic diazepam when normothermia was maintained post-ischaemically. However, post-ischaemic microinjection of diazepam into the hippocampus reduced neuronal death, but did not induce hypothermia.9 In addition, it was shown that equivalent hypothermia (to that induced by diazepam) after ischaemia did not prevent neuronal cell loss to the same extent as that found with diazepam.13 The neuroprotective action of diazepam may therefore rely on its additional mechanisms in addition to the hypothermic effects. Both enhancement of GABA neurotransmission and induced hypothermia by diazepam appear to be important in neuroprotection after ischaemia.

Benzodiazepine tolerance did not affect neuronal injury due to severe ischaemic insult, but did worsen neuronal damage when mild ischaemia was applied. This suggests that self-defence mechanisms through the GABA system are ineffective against severe ischaemia, but partially effective against mild-to-moderate ischaemia. After 8 min BCAO, rectal temperature changed similarly in naive and benzodiazepine-tolerant animals. After 6 min BCAO, the increase in rectal temperature after ischaemia seemed to be milder in naive animals. The GABA system as a self-defence mechanism might have an antihyperthermic effect against mild cerebral insult,24 and ischaemia may induce the
accumulation of GABA in the CA1 of the hippocampus. In the benzodiazepine-tolerant animals, internal accumulation of GABA might not effectively prevent exacerbation of mild ischaemic damage. Similarly, attempted external GABA stimulation by diazepam was probably inefficient in the benzodiazepine-tolerant animals, in which it was difficult to reduce severe ischaemic damage. A hypothermic effect of diazepam was not observed in these animals, which may also partially account for abolition of the neuroprotective efficacy of diazepam.

As described above, antagonism of benzodiazepine receptors by flumazenil rapidly reverses benzodiazepine tolerance. The molecular mechanism for antagonist reversal of tolerance remains obscure, but it has been proposed that, rather than directly antagonizing agonist actions, acute antagonist exposure ‘resets’ the GABA receptor to its naive, pre-treatment state. This idea is compatible with the results showing that pre-ischaemic flumazenil in benzodiazepine-tolerant animals restored the neuroprotective effect of post-ischaemic diazepam to the same degree as that of the naive animals. Moreover, further exacerbation of ischaemic neuronal damage in the benzodiazepine-tolerant animals disappeared with pre-ischaemic flumazenil treatment. Therefore, it seems that a normally functioning benzodiazepine-tolerant animals restored the neuroprotective effect of post-ischaemic diazepam to the same degree as that of the naive animals. Moreover, further exacerbation of ischaemic neuronal damage in the benzodiazepine-tolerant animals disappeared with pre-ischaemic flumazenil treatment. Therefore, it seems that a normally functioning GABA system is necessary for preventing further deterioration of neuronal injury. However, flumazenil pre-treatment damaged the neuroprotective efficacy of post-ischaemic diazepam in the naive animals. Flumazenil has an extremely short terminal half-life (<10 min). Thus, it is difficult to believe that flumazenil administered 12 h before ischaemia antagonized diazepam, and further exacerbation of ischaemic neuronal damage was not observed with flumazenil pre-treatment in the naive animals. It is possible that some toxic events induced by flumazenil in the 12 h before ischaemia may have occurred in the naive animals, including seizure activity or aberrant behaviour. Therefore, this phenomenon is not easily explained by antagonism of internal or external agonistic actions. Regardless, it is clear that flumazenil administration before ischaemia provides no benefit under conditions in which benzodiazepine tolerance is not induced.

Our study has several limitations. First, it has been shown that down-regulation of brain benzodiazepine receptors ranges from 12% to 25% after chronic flurazepam treatment. It is unclear whether this relatively small down-regulation significantly reduces neuroprotection by diazepam. However, the duration of tolerance is much longer than that reported for receptor downregulation. Different mechanisms or different neural systems must therefore mediate tolerance of benzodiazepines, besides morphological changes. Regarding the discrepancy between functional and morphological outcomes, the moderate neuroprotection in this study might be related to the failure to detect functional improvement. In particular, learning and memory may have improved and neurological assessment should have included assessment of these and motor activity, and changes may explain the reason for the discrepancy between functional and morphological outcomes. Ischaemia stimulates active responses in the brain, which are characterized by ongoing neuronal loss long after ischaemia for 5 days. Longer term effects are not known since neural effects were only studied for 5 days, and is delayed but not prevented. However, such a delay can increase the interventional (therapeutic) window for the application of other interventions. Body temperature control was not performed post-ischaemically in this study and the variation in temperatures could have obscured neuroprotective effects of diazepam or benzodiazepine tolerance. It might have been better to study the benzodiazepine contribution to consistent conditions of neuroprotection under a mild hypothermia. Lastly, the early GABAergic activation (EGASIS) trial, which is a large, multicentre, randomized, clinical trial evaluating diazepam as an early neuroprotective drug in acute stroke, failed to show neuroprotective efficacy. The neuroprotective effect of post-ischaemic diazepam might have been restored by using higher doses in benzodiazepine-tolerant animals. If so, dose adjustment based on prior history would be a useful intervention as pre-ischaemic administration of flumazenil is clearly not possible in a stroke patient.

In summary, benzodiazepine tolerance induced by chronic benzodiazepine administration can worsen ischaemic neuronal injury and abolish the neuroprotective efficacy of post-ischaemic diazepam. In contrast, pre-ischaemic flumazenil treatment can reverse benzodiazepine tolerance and restore the neuroprotective efficacy of post-ischaemic diazepam. However, flumazenil might increase ischaemic injury or reduce the neuroprotective efficacy of diazepam. Specific populations at risk for developing cerebral ischaemia may need specific management according to the benzodiazepine prescription.

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

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None declared.

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