Journal of the American Association for Laboratory Animal Science
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Vol 64, No 2
March 2025
Pages 250–254

Evaluating Three Injection Sites for Sedation/Anesthesia Using Ketamine-Xylazine in C57BL/6 Mice (*Mus musculus*): Intraperitoneal, Subcutaneous Interscapular, and Subcutaneous GV20 Acupuncture Sites

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Mice are commonly anesthetized (or sedated) with drugs injected via the intraperitoneal or subcutaneous interscapular routes. The Governing Vessel 20 (GV20) site, located at the top of the head, is an alternative subcutaneous injection route, shown to be successful in dogs and cats. In a multicenter, randomized, blinded study design, C57BL/6 mice (*n* = 66: 60 males, 6 females) were assigned to injection with ketamine (60 mg/kg) and xylazine (5 mg/kg). Injection distributions were as follows: site 1, GV20 (*n* = 10) and intraperitoneal (*n* = 9); site 2, GV20 (*n* = 16), interscapular subcutaneous (*n* = 15), and intraperitoneal (*n* = 16). Outcome measures were times to ataxia, sternal recumbency, and the loss of righting reflex. Data from sites 1 and 2 were pooled following confirmation that there were no significant differences (Mann–Whitney test). Outcome measures were compared between injection routes with a Kruskal–Wallis test followed by a Dunn multiple comparisons test. Results are reported as median (range) times. Intraperitoneal injection was faster acting than the other injection routes, and there were no significant differences between the GV20 and interscapular subcutaneous routes: ataxia (GV20, 187.0 s [120 to 272]; subcutaneous, 165.0 s [120 to 372]; intraperitoneal, 88.5 s [58 to 171]), sternal recumbency (GV20, 305.5 s [153 to 400], subcutaneous, 305.0 s [249 to 384]; intraperitoneal, 184.5 s [120 to 397]), loss of righting reflex (GV20, 399.0 s [208 to 589]; subcutaneous, 347.0 s [249 to 468]; intraperitoneal, 190.0 s [142 to 402]). In summary, the GV20 subcutaneous injection route does not appear to have benefits compared with the intraperitoneal route and is not superior to the interscapular subcutaneous injection route in C57BL/6 mice when evaluated using a combination of ketamine and xylazine.

Abbreviations: GV20, Governing Vessel 20; LORR, loss of righting reflex.

DOI: 10.30802/AALAS-JAALAS-24-127

Introduction

The goal of delivering sedative and anesthetic drugs is to rapidly achieve a useful drug concentration at the site of action, the CNS. In general, the rate of absorption for anesthetics is fastest through the intravenous route, then intraperitoneal, followed in order by intramuscular, subcutaneous, and then oral.[22]

While intravenous injection is ideal, it can be difficult to achieve consistently in laboratory rodents, especially by personnel with less training or experience.[22,23] Consequently, the intraperitoneal route of injection is widely used as an alternative. In addition to being relatively simple and quick to perform, drug absorption by the intraperitoneal route is fast.[7] However, intraperitoneal injection carries an inherent risk of misinjection, ranging from 6% to 20% in rats, and 10% to 20% in mice.[7–9] Misinjection results in a range of undesirable outcomes, from more benign effects such as delayed onset of drug action to complete absence of drug effect, to adverse effects such as visceral damage, pain, or death.[3,8,9]

Substances administered through the subcutaneous route are usually absorbed at a slower rate compared with intravenous or intraperitoneal routes and may result in greater variability in effects.[14,22] However, when compared with intraperitoneal injections, subcutaneous injections potentially cause less tissue trauma and stress and require less technical skill to master.[12,22]

A relatively recent alternative injection location to those traditionally used in laboratory and companion animals is the Governing Vessel 20 (GV20) site. GV20 is an acupuncture site associated with sedation when it is stimulated with a needle.[13,15,19] It is located on the top of the skull, along the dorsal midline and intersected by the coronal suture line connecting the rostral margins of the ears (Figure 1).[18,20] Several studies in dogs have shown that drugs (typically an α_2-adrenergic agonist with or without an opioid) injected subcutaneously at the GV20 site result in an onset of sedation that was substantially faster than with intramuscular injection and closer to the speed of effect observed with intravenous injection.[11,14,20] While the mechanism of action remains unknown, efficacy is maintained

Submitted: 03 Nov 2024. Revision requested: 09 Dec 2024. Accepted: 27 Dec 2024.
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This article contains supplemental materials online.

Figure 1. Illustration depicting needle target for GV20 injection (indicated by "X") with the box indicating the location of injectate deposition.

when drugs are injected subcutaneously adjacent to the GV20 site in dogs, suggesting that acupuncture is not the mechanism of action.[14] Recent work in cats suggests that relatively high perfusion of the subcutaneous region around the GV20 site, compared with perfusion of the interscapular subcutaneous region, may explain rapid drug absorption following injection at or near the GV20 site.[21] From a search of the literature, we found that there are no studies reporting a comparison of the GV20 injection site with other more standard injection routes in mice.[10,16,17]

The aim of this study was to compare sedation in mice following injection of ketamine and xylazine via one of 3 different injection routes: intraperitoneal, subcutaneous interscapular, and GV20. It was hypothesized that GV20 injection would result in a faster onset of sedation compared with the subcutaneous interscapular route, and comparable to the intraperitoneal route. The primary outcome measures were time from injection to ataxia, time from injection to sternal recumbency, time from injection to loss of righting reflex (LORR), and time from injection to loss of pedal withdrawal reflex.

Ethical Review

This multicenter study was approved by the Health Sciences Animal Care Committee of the University of Calgary and by the IACUC of Columbia University.

Materials and Methods

Experimental animals. Animals used at Columbia University (site 1) were as follows: 19 C57BL/6 mice, aged 9 wk, body weight 22.7 ± 2.1 g (mean ± SD), 15 C57BL/6NCrl males (Charles River Laboratories, Wilmington, MA), 4 C57BL/6J females (The Jackson Laboratory, Bar Harbor, ME). Site 1 mice were group housed with up to 5 animals per cage (NexGen 500 IVC mouse caging; Allentown, Allentown, NJ) with all caging material and bedding autoclaved. The light/dark cycle hours were 12-h light/12-h dark, with a temperature of 20 to 26 °C and 30% to 70% humidity. All cages had nesting material (Enviro-dri; Shepherd Specialty Papers, Quakertown, PA), ad libitum access to irradiated food (PicoLab laboratory rodent diet; LabDiet, St. Louis, MO), and reverse osmosis water through an automatic watering system. Health checks were performed daily. Mice were maintained under conditions to exclude hepatitis virus, minute virus of mice, mouse parvovirus, mouse rotavirus, encephalomyelitis virus, pneumonia virus of mice, Sendai virus, reovirus 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, ectromelia virus, mouse adenovirus, polyoma virus, K virus, and endoparasites/ectoparasites.

Animals used at the University of Calgary (site 2) were as follows: 47 C57BL/6NCrl mice, median age 18 wk (range 3 wk to 7 mo), body mass 29.0 ± 4.0 g, 44 males, 3 females, supplied from the in-house breeding colony. Animals were housed in groups of 2 to 5 (IVC Greenline cages; Tecniplast, Buguggiate, Italy) with aspen chip bedding. The light/dark cycle hours were 12-h light/12-h dark, with a temperature of 20 °C to 22 °C and humidity of 40%. All cages had 2 pieces of enrichment (for example, crinkle paper, paper domes, house/domes [InnoDome; Bio-Serv, S3174]). There was ad libitum access to food (Pico-Vac mouse diet 20, 4.5% fat; LabDiet, St. Louis MO) and water (reverse osmosis, rechlorinated, pH approximately 7; Avidity automatic watering system). Health checks were performed daily. Mice tested negative for Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse polio virus, reovirus 3, lymphocytic choriomeningitis virus, hantavirus, ectromelia virus, mouse adenovirus, polyoma virus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, mouse parvoviruses, *Mycoplasma pulmonis*, *Encephalitozoon cuniculi*, *Filobacterium rodentium*, pinworms, and fur mites.

The difference in numbers of animals at each experimental site reflected a focus on using animals available in-house (site 2) to control project costs while still allowing the goal of a multicenter study to be achieved.

Sample size. A sample size estimation was performed using G*Power software, with an estimated sample size of approximately 13 animals that was based on 90% power and an α value of 0.05 to detect a difference of 60 s (SD, 45 s) between different injection routes.[5]

Study design. Order of animal use was block randomized (RANDOM.ORG). At site 1 there were 2 treatment groups: intraperitoneal or GV20. The subcutaneous interscapular route was selected to serve as a subcutaneous injection control for the GV20 route.

At each site, 2 researchers calculated the required drug doses and injected the mice, while a third researcher, responsible for assessing sedation, was blinded to the injection route.

Experimental procedures. ***Sedation protocol.*** Drug doses were selected based on pilot data, with the goal of achieving heavy sedation/light anesthesia to allow identification of outcome measures (ataxia, sternal recumbency, LORR).

The same doses were used for all injection routes: ketamine (60 mg/kg, ketamine hydrochloride, 100 mg/mL, Dechra Veterinary Products, Overland Park, KS, or 100 mg/mL, Narketan, Vetoquinol, Lavaltrie, QC, Canada) and xylazine (5 mg/kg, 100 mg/mL, Covertus North America, Dublin, OH, or 100 mg/mL, Nerfasin 100, Dechra Regulatory, Bladel, the Netherlands).

Drugs were diluted with 0.9% saline (ketamine, final concentration 60 mg/mL; xylazine, final concentration 5 mg/mL) before combination into a single syringe for injection (29-gauge insulin syringe [UltiMed, Excelsior, MN]). At site 2, methylene blue dye (0.01 mL) was added to each syringe before injection to allow for identification of misinjection.

Injection and assessment. For intraperitoneal injections, a single investigator performed restraint and injection. For subcutaneous interscapular and GV20 injections, one investigator performed restraint while the other performed injection. The GV20 injection injectate deposition area was within the subcutaneous region delimited by the medial margins of the ears laterally, the rostral margins of the ears cranially, and the caudal margins of the ears caudally, with the needle inserted at midline within this area (Figure 1). Immediately after injection, each mouse was placed in an empty observation cage that sat on top of a warming pad.

Sedation assessment was performed by a single investigator who was blinded to treatment. The following outcomes were used to assess sedation: time from completion of injection to ataxia, time from completion of injection to sternal recumbency,

time from completion of injection to loss of righting reflex, and time from completion of injection to loss of hindlimb pedal reflex.

Ataxia was defined as the moment the lateral surface of a thigh contacted the cage floor, or when the caudoventral abdomen of the mouse was in full contact with the cage floor. Sternal recumbency was defined as the ventral thorax, neck, and mandible in full contact with the cage floor. Immediately after identifying sternal recumbency, the mouse was placed manually into dorsal recumbency to test the righting reflex, with LORR defined as remaining in dorsal recumbency for 15 s with no paws touching the floor. If LORR occurred, the pedal withdrawal reflex was tested by applying pressure across a digit with toothed forceps. LORR (with and without pedal withdrawal reflex) was tested every minute after sternal recumbency for 5 min. If a mouse never achieved sternal recumbency by 7 min after injection, the observation was ended. Once fully recovered, mice at site 1 were returned to their home cages and later transitioned to the institutional training protocol. Mice at site 2 were euthanized at the end of the observation period by cervical dislocation, preceded by isoflurane anesthesia if sedation was considered insufficient. Mice at site 2 were necropsied after euthanasia to identify instances of misinjection.

Exclusion criteria. It was established *a priori* that mice having suspected misinjection (based on absence of sedation) at site 1 or with confirmation of misinjection at site 2 would be removed from the data analysis. Mice at site 2 were necropsied following euthanasia, and animals with evidence of misinjection were excluded from analysis.

Statistical analysis. Data were analyzed using commercial software (GraphPad Prism version 10.2.3, for macOS, GraphPad Software, San Diego, CA). Normality was assessed with a Shapiro–Wilk test.

To assess whether there were differences between site 1 and site 2, times to ataxia, sternal recumbency, and LORR were compared for each injection route between sites with a Mann–Whitney test. The subcutaneous interscapular route was not included, as it was only performed at site 2. If no significant differences in outcome were identified between the different sites, site data were pooled and the effects of injection route on each outcome were compared with a Kruskal–Wallis test followed by a Dunn multiple comparison test (each route compared against the other).

Values of $P < 0.05$ were considered significant. Data are presented as mean ± SD or median (range). The 95% CIs for mean or median difference are presented where available. Raw data used to generate results are available in a repository: https://doi.org/10.7910/DVN/OJ5VI4.

Results

For site 1, there were 9 mice in the intraperitoneal treatment group (7 male, 2 female) and 10 mice in the GV20 group (8 male, 2 female). At site 2 there were 3 treatment groups: subcutaneous interscapular with 15 mice (14 male, 1 female), intraperitoneal with 16 mice (15 male, 1 female), and GV20 with 16 mice (15 male, 1 female). Combining the 2 sites, a total of 66 mice were used in this experiment, divided into GV20 ($n = 26$), intraperitoneal ($n = 25$) or subcutaneous interscapular ($n = 15$) treatment groups. The following animals were excluded: site 1, no animals excluded; site 2, 3 animals were excluded (1 intraperitoneal male, 1 subcutaneous interscapular male, 1 GV20 female). The 3 excluded animals were due to misinjections that were confirmed at necropsy. No animals from site 1 were excluded. This resulted in data from 63 mice included for analysis.

Loss of pedal reflex only occurred in 2 of 63 animals (2 male mice, both intraperitoneal injection, 296 s and 425 s to loss of pedal reflex), so these data were not analyzed.

Outcome data were similar between sites for the GV20 and intraperitoneal injection routes (no statistically significant differences for each outcome measure; see Supplementary Materials); therefore, data were pooled for further analysis.

From the pooled site data, there were no significant differences observed between the GV20 and the subcutaneous interscapular groups for any of the outcome measures, but the intraperitoneal group achieved each outcome significantly faster than did either the GV20 or subcutaneous interscapular groups (Figures 2–4). The difference in median times to achieve each outcome was approximately 100 to 200 s faster in the intraperitoneal group.

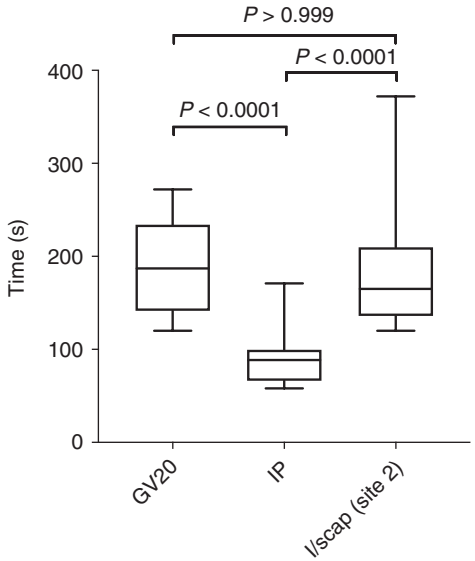

Figure 2. Median time from injection to ataxia for mice with error bars showing 95% CI, separated by injection route. GV20, Governing Vessel 20; IP, intraperitoneal; I/scap, subcutaneous interscapular.

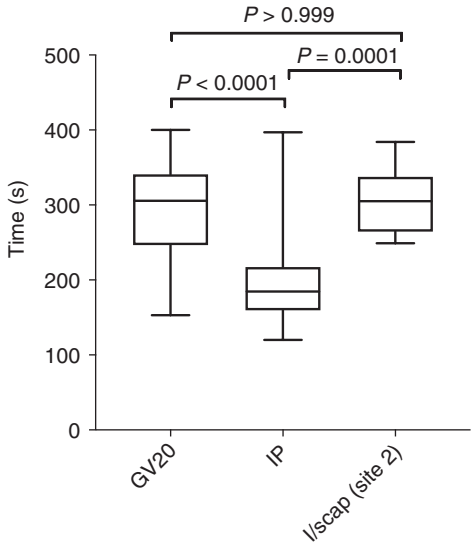

Figure 3. Median time from injection to sternal for mice with error bars showing 95% CI, separated by injection route. GV20, Governing Vessel 20; IP, intraperitoneal; I/scap, subcutaneous interscapular.

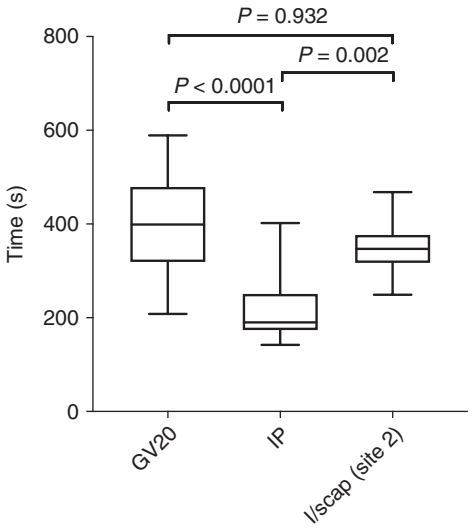

Figure 4. Median time from injection to loss of righting reflex (LORR) for mice with error bars showing 95% CI interval, separated by injection route. GV20, Governing Vessel 20; IP, intraperitoneal; I/scap, subcutaneous interscapular.

Discussion

In the current study, the efficacy levels of intraperitoneal, subcutaneous interscapular, and GV20 injection routes for inducing sedation in CB57BL/6 mice were compared through the use of a ketamine/xylazine drug combination. The intraperitoneal injection route induced sedation significantly faster than did both subcutaneous interscapular and GV20 injection routes.

In contrast to the results of the current study, sedative drug delivery using the GV20 subcutaneous injection route has been reported to achieve improved levels of sedation in dogs and cats when compared with traditional injection routes (intramuscular and intravenous).[11,20,21] In dogs, a sedative drug combination (α_2-adrenergic agonist with and without opioid) delivered by the GV20 subcutaneous route was more effective than intramuscular injection, resulting in a faster and deeper onset of sedation.[11,20] Interestingly, GV20 subcutaneous injection compared favorably to intravenous injection when assessing sedative effects in dogs.[11] While intravenous injections were not performed in the current study, the results from GV20 subcutaneous injection in dogs are in contrast with the intraperitoneal results observed in the current study, as both usually have a higher rate of absorption than intramuscular injections.[22] Absorption of drugs by the intraperitoneal route is generally fast, making it suitable for situations when rapid onset is desirable, such as for euthanasia.[7,8,24] Therefore, based on the canine literature, it was expected that the GV20 subcutaneous route would result in onset times closer to those of intraperitoneal injections than interscapular subcutaneous injections.

In cats, a comparison of a sedation protocol (α_2-adrenergic agonist and an opioid) with injection adjacent to the GV20 subcutaneous site, intramuscular (lumbar epaxial) injection, or interscapular subcutaneous injection found that sedation was superior with GV20 subcutaneous and intramuscular injection compared with interscapular subcutaneous injection.[21] These results differ from those of the current study and are closer to results observed in dogs.

The interspecies differences in efficacy of the GV20 subcutaneous route cannot be explained by the results of the current study. Notably, the mechanism of action associated with GV20 subcutaneous injection has received little attention. While originally attributed to an acupuncture effect, subcutaneous injection adjacent to the GV20 acupuncture point appears to generate equivalent effects, suggestive of a non-acupuncture mechanism.[14,20] Using infrared thermography, recent work in cats found that temperatures over the GV20 subcutaneous region were significantly higher than those over the interscapular subcutaneous region. This temperature difference suggests that blood perfusion is greater over the head than the interscapular region, which offers an explanation for the sedation differences observed in cats as a result of greater drug absorption.[21] Given the efficacy of GV20 subcutaneous drug delivery in dogs, a similar mechanism of action is possible. In contrast, the results of the current study may reflect minimal differences in perfusion between the interscapular subcutaneous and GV20 subcutaneous sites in mice. Further work is needed to confirm this.

The doses of ketamine (60 mg/kg)/xylazine (5 mg/kg) used in the current study were not intended to replicate those that might be used for induction and maintenance of anesthesia for experimental surgical procedures.[1,2,4,6,12] While pedal reflex was tested in this study, the dose used was not adequate to consistently induce loss of pedal reflex (2 out of 63 mice). The ketamine/xylazine doses selected were intentionally low to better track the progression of mice to sedation before LORR, which was usually the first visible sign of sedation in other mice studies.[6,12] The total volume of injection in the present study, at the drug concentrations used, was approximately 0.046 mL (estimated body mass of 23 g).

The following study limitations should be considered. First, both sexes were included in the study but there was no intention to study sex differences, and the study was not sufficiently powered to analyze data by sex. Second, the study was limited to a single mouse strain. Third, drugs studied were limited to a single dose of ketamine/xylazine.

In conclusion, the GV20 subcutaneous injection route does not appear to have benefits compared with the intraperitoneal route and is not superior to the interscapular subcutaneous injection route in C57BL/6 mice when evaluated using a combination of ketamine/xylazine.

Supplementary Materials

Figure S1. Time to ataxia between testing sites 1 and 2. No significant differences were observed for the GV20 injection route (95% CI of median difference, −16.0 to 68.0 s) or intraperitoneal (IP) injection route (95% CI of median difference, −8.0 to 34.0 s).

Figure S2. Time to sternal between testing sites 1 and 2. No significant differences were observed for the GV20 injection route (95% CI of median difference, −84.0 to 24.0 s) or intraperitoneal (IP) injection route (95% CI of median difference, −27.0 to 57.0 s).

Figure S3. Time to loss of righting reflex (LORR) between testing sites 1 and 2. No significant differences were observed for the GV20 injection route (95% CI of median difference, −124.0 to 93.0 s) or intraperitoneal (IP) injection route (95% CI of median difference, −15.0 to117.0 s).

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding

This work was funded by the Natural Sciences and Engineering Research Council.

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