Journal of the American Association for Laboratory Animal Science
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Vol 61, No 2
March 2022
Pages 173–180

Single-dose Diazepam Administration Improves Pairing Success of Unfamiliar Adult Male Rhesus Macaques (*Macaca mulatta*)

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Social housing is one of the best forms of environmental enhancement for nonhuman primates, and current research into pair compatibility and introduction techniques focuses on improving safety and outcome. The gradual steps method (GS), which is widely used for introducing indoor-housed macaques, involves an initial phase of limited physical contact to allow animals to acclimate to one another prior to full contact. A safer, more efficacious introduction method is needed. The administration of diazepam, a sedating anxiolytic medication, is known to increase affiliative behavior in familiar, socially housed rhesus macaques. We hypothesized that administration of a single dose of diazepam prior to full contact introduction without a protected contact phase would improve the success rate of isosexual introductions of unfamiliar macaques as compared with the success rate of GS. We administered 3.2 mg/kg oral diazepam to 34 adult male rhesus macaques (*Macaca mulatta*) 30-45 min prior to introduction into full contact. Pairs were deemed successful after 14 consecutive days of compatible full-contact housing. Behavioral data collected during these introductions was compared with data collected on 58 adult males during social introductions using GS. Sixteen of 17 introductions (94%) employing diazepam were successful. This success rate was significantly higher than the 45% success rate of introductions using GS. We also found that a longer duration of single housing and increased age were predictive of pair failure in animals introduced using GS. Our results suggest that diazepam administration prior to full contact introductions increases the success rate of male social introductions.

Abbreviations: GS, gradual steps; PC, protected contact; FC, full contact; Tulane National Primate Research Center, TNPRC

DOI: 10.30802/AALAS-JAALAS-21-000059

Introduction

Socially housing nonhuman primates (NHP) improves welfare, and thus *The Guide for the Care and Use of Animals*[13] recommends that NHP be socially housed unless they have medical or experimental exemptions or have been deemed socially incompatible. Rhesus macaques (*Macaca mulatta*) are one of the most commonly used NHP in biomedical research, and extensive investigations have been performed on environmental enhancement methods to improve their welfare.[12,28] The primary goals of environmental enhancement for laboratory animals are to improve welfare, increase species-typical behavior, and decrease abnormal behavior.[28] Macaques in established pairs exhibit fewer abnormal or self-injurious behaviors than do singly housed counterparts.[3,27] Social housing is the most effective form of environmental enhancement for indoor-housed research macaques, allowing compatible animals to interact in a positive manner, engage in species-typical behavior such as grooming or huddling,[19,28,41] and exhibit lower fecal cortisol levels compared with singly housed individuals.[18]

Despite the established benefits of social housing, macaque social introductions are inherently risky and likely to generate stress. The dominance style of rhesus macaques is characterized as despotic in that they may resort to aggression and wounding to establish and maintain the rank hierarchy within a pair or group.[6,15,29,38,43,45] Some studies report that traumatic wounding is more common in outdoor, socially housed, adult males than in females.[45] In practice, serious wounding from male macaques' large canine teeth is a concern; this concern may limit attempts to pair house males indoors.[17,36] Considerations for pair formation such as temperament testing[11] and identifying animals of disparate weights[8] contribute to increased pairing success rates and less frequent wounding. In our experience, multiple previous failed social housing attempts, high aggression, or prolonged single housing correlate with lower pairing success rates.

Different introduction methods have been developed to improve pair housing success. A previous review[44] outlined limitations associated with 5 introduction methods including rapid steps, cage-run-cage, transport, anesthetization, and gradual steps (GS). First, the rapid steps method allows individual macaques to have visual contact through a mesh or clear acrylic partition prior to introduction into full contact (FC; housing in a shared space) and may cause short-term distress (for example, because the animal has no control of proximity at first social access). Second, the cage-run-cage method employs visual and protected contact (PC; housing with a barrier permitting social contact but not entry into partner's cage) phases prior to the FC introduction in a larger, novel environment. However, this method may create a false sense of security in staff because some behaviors, including aggression, may not be evident until the FC introduction phase. Extra space is desirable for high-risk introductions; however, the increased personnel time and housing space requirements negatively

Received: 25 May 2021. Revision requested: 20 July 2021. Accepted: 17 Dec 2021.
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impact feasibility at most institutions.[35] Third, the transport method, which involves movement of animals in transport cages within or between facilities, has been used for FC introductions. This method causes a significant amount of stress for animals and presents the same risks as the rapid steps introduction method.[24] Fourth, the anesthetization method allows unfamiliar individuals to recover in FC with a new social partner but risks the individuals awakening at different times.[7,30] Fifth, during a GS introduction, animals in adjacent cages are introduced into either visual contact through a clear panel or directly into PC employing a solid barrier with holes or bars that allow limited tactile contact. Different institutions have different standards for behaviors that must be observed before introducing animals into FC. The disadvantages of the GS method are similar to those of the cage-run-cage method except that a large introduction space is not required.[44] Often, the method of introduction is chosen based on species, individual characteristics such as age and prior social history, available time and facility space capacities, and experience of the behavioral staff.

The Tulane National Primate Research Center (TNPRC) and many other institutions housing NHP employ the GS introduction method previously described.[2,44] Individuals remain in PC until staff are confident that they are not displaying signs of overt aggression or fear. If behaviors of concern are not observed during the PC phase, the separating partition is removed, and animals are monitored for compatibility during the FC introduction.[4,18,47] Unfortunately, drawbacks to the GS method include 1) the lack of interaction exhibited by some pairs during PC that makes it difficult to determine when individuals can be safely introduced into FC and 2) the apparent frustration that some individuals seem to experience due to their inability to interact in species-typical ways during PC. Anesthetized introductions address some of the drawbacks of the GS method such as the potential development of frustration while in PC. However, disparate recovery times may put paired animals at risk for injuries due to an inability to defend themselves and to appropriately respond to social cues during anesthetic recovery.[44] To ensure the safety of the animals, anesthetized pairs require constant monitoring during sometimes prolonged recovery; because of practical considerations, this requirement can be difficult to incorporate into many pairing protocols.

Reports of successful social introductions after ketamine administration prompted our interest in developing a novel method of pharmacological intervention that would improve success rates of pair introductions, address the drawbacks of previously discussed introduction methods, and promote the wellbeing of the animals. We considered different drug classes that might overcome the limitations of anesthetized introductions and opted to assess the effect of benzodiazepine administration on social introduction outcomes. Benzodiazepines are GABA receptor positive allosteric modulators that act as anxiolytics and may cause sedation at higher doses.[9,32,40] Benzodiazepines including diazepam have anticonflict effects in rhesus monkeys in a model used to predict anxiolytic effects of drugs in humans.[20,39] Diazepam is a longer-acting benzodiazepine that has been safely used in NHP and has been shown to have sedating and anxiolytic effects.[5,33] Paradoxical effects of diazepam such as anxiety and aggression have been reported in rodents,[21,31] companion animals,[23] and humans,[22] but little is known about what factors predispose individuals to an aggressive response. Although diazepam has not been previously used in NHP to facilitate introductions, research has shown other positive behavioral effects of diazepam administration such as reduced fear-potentiated startle in macaques and prolonged duration of greeting behavior in pairs of juvenile rhesus macaques.[42,46] A previous study[16] investigated the behavioral effects of diazepam in settled pair-housed rhesus macaques and demonstrated that doses of diazepam as low as 0.1 mg/kg decreased aggressive behavior while doses greater than 3 mg/kg led to sedation, more pronounced submissive behavior, and decreased partner-directed aggression without changing the social hierarchy of paired animals. In addition, the results of another study[25] demonstrated that administration of 5 mg/kg diazepam to individuals in settled groups of rhesus macaques increases affiliative behavior and decreases aggressive behavior in familiar, group-housed rhesus macaques.

In the current study, we conducted pair introductions after administering a 3.2 mg/kg dose of diazepam (referred to as the 'diazepam method') and compared the outcomes to retrospective data from GS introductions conducted at the TNPRC. We hypothesized that social introductions of unfamiliar, adult, male macaques after administration of 1 dose of diazepam prior to the introduction would lead to higher rates of success, increased affiliative behavior, and reduced anxiety as compared with GS introductions. We also investigated the role of individual animal characteristics in influencing outcomes of adult male pairings that used the GS introduction technique as well as the differences in group composition between the GS and diazepam groups.

Materials and Methods

Animals. The macaques used in this study were housed at the Tulane National Primate Research Center, an AAALAC-accredited facility. This study was performed in accordance with the *Guide for Care and Use of Laboratory Animals*[13] and the Animal Welfare Act after protocol approval by TNPRC's Institutional Animal Care and Use Committee (IACUC). Ninety-two Indian-ancestry, male, singly housed, adult, rhesus macaques from the TNPRC breeding colony were enrolled in this study. All animals on the study were seronegative for *Macacine alphaherpesvirus* 1, simian immunodeficiency virus (SIV), and simian T-cell lymphotropic virus (STLV-1), and seronegative and virus negative for simian betaretrovirus (SRV). Thirty-four subjects introduced from January 2020 through July 2020 were assigned to the diazepam group. Findings from this experimental group were compared with retrospective data from 58 individuals introduced using the GS method as part of the TNPRC's regularly implemented environmental enhancement plan. At the time of enrollment, the diazepam subjects ranged in age from 4.8 to 15.0 y (mean = 8.2 y) and weighed between 6.5 and 17.6 kg (mean = 12.3 kg). The GS subjects ranged in age from 4.0 to 16.7 y (mean = 6.9 y) and weighed between 4.9 and 17.5 kg (mean 10.6 kg). Macaques that received diazepam had been singly housed indoors from 76 to 653 d with a mean of 194 d, and GS macaques had been singly housed indoors from 14 to 525 d with a mean of 137 d. Eleven of the 34 diazepam subjects and 10 of 58 GS subjects spent at least 2 mo in the nursery and 2 mo with their dams during the first year of life.

Subjects were housed in interconnecting stainless steel NHP cages that exceeded the minimal standards set forth by the USDA and *The Guide for Care and Use of Laboratory* Animals.[13] Individuals that weighed less than 10 kg were individually housed in a cage with 0.4 m^2 floor space and 0.9 m vertical space, and macaques weighing 10 to 20 kg had access to 2 side-by-side cages, providing 0.8 m^2 of floor space and 0.9 m of vertical space. Integrated cage racks were composed of 2 tiers with 2 cages on each level. The design allows several cage racks to be connected to permit the horizontal connection of multiple cages. Each cage was outfitted with foraging device(s), perches, and various manipulanda both installed on and loose within the cage. Rooms were maintained on a 12:12-h light: dark cycle within a temperature range of 18° to 22 °C (64° to 72°F) and a relative humidity range of 30% to 70%.

Subjects were fed a standard commercial NHP diet (Lab Diet Fiber-Plus Monkey Diet, St. Louis, MO) twice daily with ad libitum access to water via an on-demand water valve. Subjects received forage, fresh fruit and/or vegetables, and other food items at least 5 times per week in accordance with the TNPRC Environmental Enhancement Program.

Partner selection and group assignment. Potential pairs were selected from indoor, singly housed adult males raised in the TNPRC breeding colony. Most individuals on this project had previously been removed from the breeding colony for clinical treatment. After the clinical cases were closed, the macaques were pair-housed for this study while awaiting return to the breeding colony or assignment to research. Pair assignment was based on lack of prior familiarity and disparate weights when possible, based on prior research establishing higher success rates in pairs with greater weight differences.[8] Temperament data was not available for most subjects and was not used in partner selection. Individuals were paired using either GS introduction or FC introduction after diazepam administration. Prior to pairing, physical examinations were performed, and complete blood count and blood chemistry results were reviewed for macaques in the diazepam pairing group to ensure that they were all in good health.

Diazepam suspension preparation. For the oral suspension, diazepam (19.2 mg/mL) was formulated by adding 100 crushed diazepam tablets (10-mg tablets; Covetrus, Dublin, OH) to 18 mL Ora Plus (Minneapolis, MN), 25 mL Ora Sweet (Minneapolis, MN), and 33 drops of raspberry flavor syrup (FlavorRx, Columbia, MD).

Prior to introduction, each subject in the diazepam group received a single 3.2 mg/kg dose of oral diazepam in a high-value food item and was monitored for behavioral changes suggestive of drug effect to determine the expected response to this dose of diazepam.

Gradual steps introductions. Introductions were conducted in accordance with TNPRC standard practices for social housing. Subjects were moved into adjacent cages separated by solid partitions for at least 3 d before introduction into PC. During the initial phase of introduction, individuals were placed in PC by replacing the solid partition separating them with a divider. Dividers were either stainless steel partitions with multiple 2 to 3-in holes or vertical bars spaced 3 cm apart to allow limited physical contact. Pairs were observed and behavioral data were collected in 10-min sessions using 1/0 (yes/no) scoring with a 30 s intersampling interval after introduction into PC. All observations were performed by the same behavioral technician, who was unfamiliar to all subjects in both the GS and diazepam groups. Ten min of data were collected, followed by additional monitoring as needed. Data collection was terminated, and macaques were separated before the completion of the session if overt aggressive behavior or wounding were observed. The decision to terminate introductions was based on prior experience and criteria established by the TNPRC Unit of Behavioral Management such as persistent or escalating aggression or persistent fear. Pairs separated during PC were considered failures. The duration of PC ranged from 5 to 27 d based on the nature of behaviors observed in person or on video. The decision to move forward with FC introductions was based on the prevalence and patterns of behaviors such as aggression, affiliation, or distress. When subjects exhibited concerning behavior, video footage was collected in the absence of an observer and assessed for aggressive, agonistic, or fearful behavior that may have been suppressed in the presence of human observation.

If potential pairs were successful in PC, they were introduced into FC by removal of the divider. The same observational and behavioral assessments used for PC were used for the FC introduction. Macaques were monitored after the initial introduction according to TNPRC standard FC introduction procedures, which include behavioral data collection by direct observation and video recording.

Pairs were considered successful after 14 d of FC housing without the need for separation.[17,44] One compatible pair was successfully integrated as a pair into a larger group setting on day 13, and this pair was also categorized as successful.

Diazepam introductions. Macaques in the diazepam introduction group were moved into adjacent cages separated by solid partitions for at least 3 d prior to pairing. On the morning of the introduction, a video camera was set up and recording began before the administration of diazepam and continued for at least 3 h during and after the introduction. Each individual received a single 3.2 mg/kg dose of oral diazepam given in a high-value food item. The macaques were introduced into FC by removing the solid partition separating them 30 to 45 min after both individuals had ingested the diazepam. Pairs were monitored in person by an unfamiliar behavioral technician and the PI for at least 20 min after introduction and by video for an additional 2 h. Introductions were terminated before the completion of the initial 20-min period if overt aggressive behavior was observed. The decision to terminate introductions was based on criteria typically used by the TNPRC Unit of Behavioral Management. After introduction, pairs were assessed daily for the first 2 d and then at least weekly to monitor for agonistic behavior that would require separation. Each pair was also monitored closely in person and, for some macaques, via videotape at multiple time points to monitor compatibility.

Data collection. Behavioral data was collected via 1/0 sampling. Behavioral observations were collected during the initial 10 min after the introduction using the TNPRC ethogram for pair introductions, which includes the following behaviors (see Table 1): abnormal behavior, aggressive contact without wounding, aggressive contact with wounding, anxiety, cothreatening, displaying, fear grimacing, grooming, lip-smacking, mounting, playing, rump presenting, and threatening. Lip-smacking, mounting, rump presenting, cothreatening, and grooming were grouped into "affiliative behaviors" for analysis, and aggressive contact without wounding, aggressive contact with wounding, displaying, and threatening were grouped into "aggressive behaviors" for analysis. Abnormal behavior, fear-grimacing, and playing were excluded from the analysis because they were either not observed or seen too rarely to include in the statistical model. Data were collected on the first 10 min of the introduction in real time for the GS group and via videotape for the diazepam group. The full 10 min of data were not available for a total of 26 introductions either due to technological malfunctions or pair separation, and these macaques are excluded from the behavioral analysis. Complete behavioral data were available for 28 of 34 subjects in the diazepam group, 46 of 58 subjects in the PC phase of the GS group, and 26 of 34 subjects in the FC phase of the GS group.

Statistical analyses. Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) for Windows, Version 27.0. Results were considered significant with an $\alpha \leq 0.05$. Suc-

Table 1. Ethogram of behaviors used during observations. Note that fear grimacing, playing/play soliciting, and abnormal behavior were observed too infrequently to include in data analysis.

Agonistic Behaviors	
Aggressive contact/biting	Physical contact involving biting or biting attempts
Aggressive contact/no biting	Physical contact without involvement of the mouth (for example pushing, pulling, grabbing, and scratching)
Displaying	Vigorous shaking, slamming, or bouncing off of the cage
Fear grimacing	Grin-like facial expression involving retraction of the lips, exposing teeth
Threatening	At least one of the following partner-directed gestures: ears flattened against the head, brow retracted, open-mouth stare, head bobbing, slap surface or slap at the partner without making contact, and lunging (high-speed aggressive intention movement toward another animal)
Affiliative Behaviors	
Cothreatening/solicit cothreat	Alternating threats and glancing at the partner, who may or may not join in the threatening
Grooming	Manipulating, brushing, or licking of fur (or eyes, wounds) of another animal with the mouth and/or both hands. Includes both groomer and animal receiving grooming
Lip-smacking	Bringing the lips together rapidly, resulting in a smacking sound; teeth are covered. Directed at potential partner
Mounting	Common usage, with or without pelvic thrusting and penetration and with or without foot clasp. Includes both mounter and animal being mounted.
Playing/play soliciting	Nonaggressive, lively actions performed with another individual with or without direct physical contact (for example chasing), without pilo-erection, but with relaxed facial expressions
Rump presenting	A posture involving a stance on all fours with the hind quarters elevated and the tail raised. In some animals the tail may be lifted to the side rather than raised. In some instances, animals may place their heads between their legs. Rump presents may be accompanied by brief tail flicks. Directed at potential partner.
Other Behaviors	
Abnormal	Animal performs species atypical behaviors (for example hair plucking, self-directed, or locomotor stereotypies)
Anxiety	Body shuddering, scratching, yawning, or teeth grinding

cess rates in the diazepam administration and GS groups were compared using the Fisher's exact test. Differences between behaviors exhibited by the diazepam and GS groups were assessed using Mann-Whitney *U* tests.

To look for confounding factors in our data that may have predisposed the diazepam or GS group toward successful pairings, differences between 5 individual characteristics were analyzed: time since last social housing, age, age difference, weight, and weight difference. Data are presented as the mean and standard error of the mean. Welch's *t* tests were used to compare individual characteristics between the diazepam method and the GS method. The Levene's test for inequality was used to test for unequal variances between the groups, and results were reported accordingly.

Regression analysis to identify the characteristics or behaviors predicting pair success could not be performed for the diazepam method due to the scarcity of failed introductions. A backward step-wise binary linear regression was used to determine which individual characteristics were predictive of pair success in the GS group. All factors were initially entered into the model, and each term's contribution to the model was assessed by the goodness of fit of the model. Terms were removed if they did not improve the goodness of fit of the model. The factors found to be predictive of success in the GS group were compared with the factors found to differ between the GS and diazepam groups to further assess whether differences in characteristics between groups could influence group success rates.

Results

Introduction outcomes. Subjectively, at the onset of introduction after diazepam administration, some macaques appeared less active and slightly unsteady during ambulation, but all animals exhibited social behavior during the first 10 min after introduction.

The success rate for pairs introduced after diazepam administration (94%) was significantly higher than the overall success rate of GS introductions (45%; $P = 0.001$). Sixteen of 17 adult male FC pair introductions after diazepam administration were successful. One successful male pair exhibited aggressive contact without wounding during the initial introduction. The single male pair that failed fought immediately upon introduction, and both individuals required veterinary care for wounding. Thirteen of 29 of the GS introductions were successful. Two individuals in the GS group received wounds requiring partner separation while in FC, and 1 of these individuals required veterinary care for wounding.

The different overall success rates of the diazepam and GS groups were driven by the outcomes of the PC phase of the GS introduction method. The success rate for the PC phase in the GS method (59%, 17 of 29 introductions) was significantly lower than for the diazepam introductions (94%) ($P = 0.016$). Of the 17 successful PC introductions that occurred during the GS process, only 4 pairs failed upon introduction into FC (76% success rate). The success rate of this final phase of the GS method was not significantly different from the diazepam introductions (59% and 94%, respectively) ($P = 0.33$).

Behavior during introductions. The frequency of anxious, aggressive, and affiliative behaviors showed no significant differences between the diazepam group and the PC phase of the GS method (Table 2). Furthermore, no significant differences were found in the frequency of anxious, aggressive, and affiliative behaviors between the diazepam group and the FC phase of the GS group (Table 2).

Differences in individual characteristics between the diazepam and GS subjects. An independent samples *t* test indicated that time since last social housing was significantly higher for the diazepam group (194 ± 225 d) than the GS group (137 ± 14 d) (Table 3). Age was significantly higher in the diazepam (8.2 ± 0.4 y) than the GS group (6.9 ± 0.3 y) (Table 3). Individual body weights were also significantly higher in the diazepam (12.3 ± 0.5 kg) than the GS group (10.6 ± 0.3 kg) (Table 3). Weight differences between cage mates were also significantly greater

Table 2. The Mann-Whitney U test demonstrated that there were no significant differences in anxious, aggressive, and affiliative behaviors between the diazepam group and each phase of the GS method.

	Mann-Whitney U	*P*	*DZP (mean ± SE)*	*GS (mean ± SE)*
Diazepam compared with PC				
Anxiety	505.0	0.12	0.18 ± 0.041	0.29 ± 0.043
Aggression	553.5	0.26	0.02 ± 0.008	0.02 ± 0.006
Affiliation	475.0	0.05	0.05 ± 0.012	0.03 ± 0.006
Diazepam compared with FC				
Anxiety	278.5	0.13	0.18 ± 0.041	0.11 ± 0.027
Aggression	283.5	0.06	0.02 ± 0.008	0.003 ± 0.002
Affiliation	278.0	0.13	0.05 ± 0.012	0.03 ± 0.009

The mean and standard error of anxious, aggressive, and affiliative behaviors during diazepam and GS introductions are included.

Table 3. Duration of single housing, age, weight, and weight difference from social partner were found to be significantly different between the diazepam and gradual steps groups while age difference from social partner was not significantly different.

	t	*df*	*P*[b]	*DZP (mean± SE)*	*GS (mean± SE)*
Duration single housing (days)	2.34	90	0.02	194.2 ± 22.1	137.0 ± 13.6
Age (years)	2.31	90	0.02	8.2 ± 0.4	6.9 ± 0.3
Age difference (years)	1.64	90	0.11	2.9 ± 0.4	2.2 ± 0.3
Weight (kg)	2.73	90	0.01	12.3 ± 0.5	10.6 ± 0.4
Weight difference (kg)	5.80	90	0.00	4.8 ± 0.3	2.8 ± 0.2

b Equal variances assumed in *t* test

The mean and standard error of the mean were compared for the duration of single housing, age, weight, and weight difference from social partner the diazepam and gradual steps groups.

Table 4. Longer tenure in single housing and greater age were predictive of pair introduction failure using the gradual steps introduction method while age difference, weight, and weight difference were not predictive of pair outcome.

	B	*S.E.*	*Wald* χ^2	*df*	*P*	*Odds ratio*
Duration single housing (days)	−0.01	0.00	4.31	1	0.04	0.99
Age (years)	−0.29	0.14	4.41	1	0.04	0.75

Table 5. The mean and standard error of the mean of the duration of single housing, age, weight, and weight difference from social partner between successfully and unsuccessfully paired animals in the gradual steps groups were used to determine which characteristics were predictive of pair outcome.

	Successful		*Unsuccessful*	
	Mean	SE	Mean	SE
Duration single housing (days)	108	12	161	22
Age (years)	6.2	0.4	7.5	0.5
Age difference (years)	2.2	0.5	2.1	0.3
Weight (kg)	9.9	0.5	11.2	0.4
Weight difference (kg)	2.8	0.3	2.7	0.3

in the diazepam (4.8 ± 0.3 kg) than in the GS group (2.8 ± 0.2 kg) (Table 3).

Individual characteristics that predicted outcome in the historical GS outcome data. In the GS method, individual characteristics that predicted pair failure better than the null model included greater time since last social housing and greater age (Table 4). This comparison could not be performed for the diazepam group because of too few failures to allow the comparison. The average duration of single housing for successfully introduced macaques (108 ± 12 d, Table 5) was significantly lower than the average for unsuccessfully introduced animals (161 ± 22 d) (Table 5). The average age of successfully-introduced animals (6.2 ± 0.4 y, Table 5) was significantly lower than the average age of unsuccessfully introduced animals (7.5 ± 0.5 y, Table 5).

Discussion

The overall goal of this project was to develop a new method that improves success rates of adult male rhesus macaque social introductions. We found that administration of 3.2 mg/kg oral diazepam prior to full contact introductions of unfamiliar adult male rhesus macaques dramatically improved pair success rates compared with the gradual steps method. The results of this study indicate that administration of diazepam prior to FC introduction is a promising alternative to existing introduction methods.

Prospective and retrospective research has investigated individual characteristics and behavioral predictors of pair success and compatibility, but fewer studies have focused on development of alternative introduction methods for improving success rates.[4,8,29,44] Previous work[38] pioneered the use of a wire partition to allow PC familiarization between potential partners prior to FC introductions. This strategy, referred to as the gradual steps or protected contact method, has since been widely adopted and adapted by institutions for pair introductions of NHP.[2,4,14] A 2014 survey of facilities in the United States found that 96% of facilities used this technique during pair introductions.[2] Introduction success rates vary between studies, method, and institutions and across species, age, and sex, with success rates for rhesus males reported to be as low as 32% and as high as 100%.[1,18] Therefore, in some cases, the success rates and efficiency of introductions can be improved, particularly for adult male pairs. This study aimed to develop a new introduction method that improves success rates and addresses the drawbacks of existing introduction methods.

We compared pair introduction success rates of unfamiliar adult male rhesus macaques after administration of a single dose of 3.2 mg/kg oral diazepam without a PC phase to standard GS introductions. The study confirmed our hypothesis that diazepam administration improves introduction success rates (94% diazepam, 45% GS), even without a PC phase. Comparison of the diazepam administration method success rate to each phase of the GS showed that diazepam administration provided significantly better success rates as compared to the PC phase. However, a significant difference in success rates was not detected between the diazepam method and the FC phase of GS because most GS pairs that failed did so during the PC phase prior to FC introductions. This is unsurprising because incompatibility is often obvious during the first few minutes of the PC phase of the GS method. Diazepam administration appears to serve the same function as the PC phase by allowing animals to become familiar with each other in a less stressful situation due to the anxiolytic effect of diazepam. Diazepam administration also addresses many of the well-recognized drawbacks of the GS method. Furthermore, diazepam administration prior to introduction into FC eliminates the PC phase and the associated anxiety exhibited by some individuals yet preserves the species-typical interactions needed to establish the dominance hierarchy.

This study also aimed to assess how diazepam administration affected the behavior exhibited by individuals during introductions as compared with the PC and FC introduction phases of GS. We hypothesized that diazepam administration would decrease anxiety and increase affiliative behavior expression as was seen in a previous study[25] of familiar group-housed rhesus macaques. While this study did not detect differences in anxious, aggressive, and affiliative behaviors, introduction outcomes are nonetheless consistent with the idea that diazepam administration facilitated the development of stable social relationships. Additional studies are required to further investigate the mechanism by which diazepam improves social introduction outcomes.

The single dose of diazepam used in this study subjectively caused sedation in some animals; this was described by the observers as increased time spent resting with eyes open or closed without interacting with the environment or partner. While these observations are consistent with previous[25] findings of decreased locomotion and increased resting with eyes closed, this effect cannot be quantitatively confirmed in the present study because the ethogram used did not measure rest time. The sedative effect of the dose used may have contributed to the greater success rate in the diazepam group compared with the GS group. This potential could be studied in future studies by modifying the ethogram to capture resting behavior.

Our study also evaluated whether differences in individual characteristics of partners could confound the findings regarding the relative success of the 2 introduction methods. We found that the length of single housing, age, weight, and weight difference were all significantly greater in the diazepam group than in the GS group. We tested for the presence of confounds in 2 ways. First, we compared the individual attributes of individuals involved in successful and unsuccessful retrospective GS introductions included in the current study. Second, we examined prior literature on predictors of the outcome of GS introductions. Greater duration since last social housing and greater age were 2 attributes associated with failure in the PC phase of GS introductions in the retrospective data. This suggests that the individual characteristics of the GS group (shorter duration since last social housing and younger age) would have made animals in this group more likely to succeed and the attributes of longer duration since last social housing and older age in the diazepam group would have made that group's introductions less likely to succeed. Another study[8] similarly found that success of male pairs is more likely when macaques are younger. They also reported that a greater weight difference is predictive of success, and this is the only potentially predictive variable that could have provided an advantage to the diazepam introductions. However, in that study, the influence of differences in weight disparity between successful and unsuccessful pairings did not affect an outcome that approached the dramatically higher pairing success rates associated with introductions using diazepam. Taken together, the contrasts between the diazepam and GS groups cannot be explained by this one advantageous confounding factor. In fact, the diazepam group was more successful despite individual characteristics expected to predispose a pair toward failure.

While social housing is an essential part of NHP behavioral management programs, a 2014 survey[2] identified significant barriers to social housing, including unavailability of social partners, protocol restrictions, clinical exemptions, behavioral exemptions, time, staff, housing, space, and concerns about negative consequences.[2] The results of this study suggest that the practical barriers to implementing social housing could be significantly mitigated by using the diazepam method. For example, the aforementioned survey found that 44% of respondents reported that time and staff availability are limitations to the success of social housing programs. This finding indicates that improving the efficiency of social introductions would improve the quality of social housing programs. The GS introduction method was initially developed to allow time for unfamiliar individuals to become familiar with one another and in some studies to allow the development of a dominance hierarchy while limiting the chance of serious wounding.[14,34,37,38] However, allowing time for initial relationship formation in this fashion also results in GS introductions requiring significant time investment into repeated, extended monitoring after macaques are placed into PC and again when placed into FC.[30] Some individuals do not interact during the PC phase, which forces decision making about moving into FC or extending the PC phase based on limited information gleaned from observations. Limited interactions during PC introductions often require additional observations, thus delaying FC pair housing. In addition, pairing may be subject to health or research limitations, which limit the periods during which pairing can be performed. Improving the efficiency of the introduction process will facilitate the social housing of a larger number of animals at any given time and for the longest duration of time. Replacing the GS method with the more effective diazepam method decreases both the length of time prior to social housing and personnel time required per pair, thus improving the efficiency of the behavioral management team and allowing them to perform more social introductions.

In addition, 41% of facilities surveyed mentioned the potential limitations of cage availability as a limitation to social housing.[2] The diazepam method may reduce this limitation. GS introductions require special caging with interchangeable acrylic and/or perforated partitions. Some facilities may not have cages with these features or may only have a small number of such cages. Retrofitting to add these features is cumbersome and can result in challenges associated with availability of a cage, its components, and sanitation. In addition to improving the success rate of social introductions, the diazepam method has practical implications that can improve the overall efficiency of behavioral management programs. The success associated with diazepam introduction can reduce the use of single housing, both because of procedural efficiency resulting in prompt pairing, but also because pairs that may be successful via a diazepam introduction may fail during a GS introduction. Individuals failing in a pair may remain singly housed long term if alternative pairing candidates are not available, or they may have to undergo multiple time-intensive and risky introduction attempts to find a compatible partner.

Finally, this study had several limitations. Data in the GS group were collected in real-time during introductions by a behavioral technician using a computer tablet, but in diazepam introductions data was collected from videos. Analysis of recorded video may have allowed technicians to record more behaviors than they would have been able to log in real time, although possibly their views of the animals may have been partially obscured by partitions during parts of the video. Next, behavioral data was not available for all study subjects. Subjects were excluded from the behavioral analysis if fewer than 10 min of data were available. Also, complete data could not be collected on individuals that were separated after fewer than 10 min of contact due to aggression or incompatibility. In these cases, the behavioral data were not analyzed, but the pairs were included in the success frequency analysis. This difference influenced apparent frequency of behaviors and impeded our ability to detect behavioral changes attributed to diazepam because behavioral data was not available for separated pairs. Another limitation of the study was that we only assessed pair success over a 14-d period before removing subjects from the study. Despite these limitations, this study demonstrates that a novel method results in a rather dramatic increase in the success rate of adult male rhesus macaque pairings.

This study was intentionally performed with unfamiliar adult male macaques because this population tends to be the most difficult to pair. However, additional research is required to determine if diazepam administration affects social introductions in other age groups, females, and other NHP species. Because we had tremendous success with our most difficult population (unfamiliar adult males), we are hopeful that the results will translate to other high-risk introductions. We recommend that the diazepam method be considered for especially difficult to pair animals such as older males and animals with a history of previous unsuccessful introductions. We urge caution when employing this method with animals that have a history of self-injurious behavior, because diazepam was associated with increases in this behavior in a subset of subjects receiving the medication as a treatment.[26] Another valuable vein of research would be a comparison of animals during the PC phase of the GS method with and without diazepam administration to further investigate the behavioral effects of diazepam. In conclusion, we encourage consideration of the diazepam method as a technique for pairing NHPs. In our study, dramatic improvement in pairing success associated with the single-dose diazepam method suggest that this approach may have wide-ranging welfare impacts on the thousands of male rhesus macaques that are introduced each year, facilitating the implementation of this most effective measure for improving the welfare of laboratory primates.

Acknowledgments

We thank Robert Blair and Gina Dobek as well as the staff of Tulane National Primate Research Center. We also thank Lawrence Williams for his assistance with statistics. The authors acknowledge the financial support of grants U42OD010568, U42OD024282, and P51OD011104.

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