

Refinements for Intra-gastric Gavage in Rats

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Summary

Intra-gastric (IG)-gavage is widely used in laboratory rats in pharmacological, toxicological and pharmacokinetic studies. This technique has been claimed to result in severe stress and a variety of complications. This study was designed to compare the stress response caused by IG-gavage with steel and teflon probes, to determine whether any habituation occurred to repeated gavaging and to find out whether the use of different administration volumes within the recommended range influenced the stress response. Telemetrically registered cardiovascular responses were used to assess the stress-producing effects. During laparoscopy, transmitters with a catheter extending into the abdominal aorta were implanted into the peritoneal cavity of male Wistar rats. IG-gavage induced a significant increase in diastolic and systolic blood pressure and heart rate, lasting for about 40 minutes. IG-gavage with a stainless steel probe induced greater changes in cardiovascular parameters. It can be concluded that teflon probes are preferable because they elicit less discomfort to the animals. Repeating the IG-gavage with a teflon probe daily evoked a decrease of all parameters on the fourth day as compared with the previous days, but this did not occur in the stainless steel group. The volume administered through IG-gavage had significant effects on diastolic blood pressure, systolic blood pressure and heart rate. Surprisingly, volumes of 2 and 4 ml / kg body weight resulted in a greater response in cardiovascular parameters than volumes of 6 and 8 ml/kg. It appears that there is a window of preferred administration volumes. A routine cage change induced an increase in diastolic blood pressure, systolic blood pressure and heart rate comparable to the changes observed after IG-gavage. In conclusion, our data indicate that use of IG-gavage with a soft teflon probe and volumes 6 and 8 ml/kg are obvious refinements for the procedure.

Introduction

Intra-gastric (IG)-gavage is a widely used method for the oral dosing of drugs in laboratory rats in pharmacological, toxicological and pharmacokinetic studies. Hence, it can be assumed that a huge number of rats are subjected to this procedure every

day. Although with correct technique, lethality associated with IG-gavage is low (*McIntyre, 2001; Rao et al., 2001*), it has been shown that seemingly fast and non-invasive technique can result in severe stress and a variety of complications (for a review see *Murphy et al., 2001*). It has been suggested that IG-gavage in rats results in a feeling that can be graded ranging from light nausea to discomfort and even pain (*Alban et al., 2001*) and it is the most stressful and technically demanding method of oral administration (*Morton et al., 2001*).

Successful and the least stressful IG-gavage depends on the experience of the technician

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(McIntyre, 2001, Rao *et al.*, 2001), the correct size and type of the probe (Wheatley, 2002), an appropriate volume (Alban *et al.*, 2001) as well as the vehicle to be injected (Brown *et al.*, 2000) during the procedure.

While the majority of technicians use metal probes, others prefer those made of softer materials, such as rubber, PVC or teflon. Both types have their advantages and disadvantages. Since, stainless steel probes possess a higher risk of causing oesophageal rupture, many technicians prefer softer materials. However, soft probes can be bitten and break and solutions administered can enter respiratory passages or broken pieces of the probe can be swallowed. In both cases the animals must be euthanized (Wheatley, 2002). Furthermore, the volumes considered safe to use and recommended in textbooks and manuals, vary from 2 ml (Bekow & Baumans, 2003) to 40 ml (Diehl *et al.*, 2001) per kg of body weight for an adult rat and seem to be based on practical experience, rather than on any scientific evidence and hence may require refinement (Alban *et al.*, 2001).

This present study has used telemetrically recorded cardiovascular responses to assess the stress-producing effects. The choice of these parameters is based on two facts. Firstly, it has been demonstrated that various forms of stress can impact on cardiovascular function, this being reflected by an increase in blood pressure and heart rate (Sharp *et al.*, 2003a, 2003b; Morton & Hau, 2003). Secondly, telemetry provides an opportunity to obtain physiological measurements from freely moving rats without interference from any attached wires, tethers or jackets (Kramer, 2001). Problems related to exit sites such as infections, irritation to the animal, and maintenance are also absent. Once the telemetry device is implanted, data can be monitored 24 hours a day without human intervention or contact while the animal remains in its home cage. Another advantage of telemetry is that makes it possible to monitor cardiovascular function without inducing additional stress artefacts (Anderson *et al.*, 1999; Krohn *et al.*, 2003).

This study was designed to compare the response caused by IG-gavage with steel and teflon probes, to determine whether any habituation occurs during repeated gavaging and to find out whether use of certain administration volumes are less stressful than others.

Materials and Methods

Ethical considerations

The study was carried out at the National Laboratory Animal Centre, University of Kuopio, Finland. The protocol of study was reviewed and approved by the Animal Ethics Committee of the University of Kuopio.

Animals and animal care

Male Wistar rats (HsdBrIHan:WIST, NLAC, University of Kuopio, Finland) weighing 310-390 g (12 weeks old) at the beginning of the study were housed in groups of three in polycarbonate (59.5 x 38.0 x 20.0 cm) open top, solid bottom cages (1354G – Eurostandard Type IV, Tecniplast, Italy) with aspen chips as bedding (chip size 4 x 4 x 1 mm, Tapvei, Kaavi, Finland). Lighting was automatically controlled on a 12 h light/dark cycle (lights on at 07.00), temperature maintained at 20±2 °C and relative humidity at 50±10 %. The animals had *ad libitum* access to pelleted food (R36, Lactamin AB, Södertälje, Sweden) and tap water in polycarbonate bottles. Rectangular aspen tubes (length 20.0 cm, height 11.0 cm, thickness of the walls 1.5 cm) were used as environmental enrichment. The tubes were placed into the cages one week before the implantation of telemetry transmitters and were replaced with new tubes once a week during every routine cage change (Fridays). One rat was chosen on a random basis from each cage for the implantation of the transmitter.

Telemetry system

The telemetry system used for the acquisition of data consisted of the following components from Data Science International, St. Paul, MN, USA: (I) PhysioTel® PA-C40 transmitters, with a weight of 9

g and a volume of 4.5 ml (*i.e.* constituting under 3 % of body mass) and configured to measure heart rate, systolic and diastolic pressure and locomotor activity; (II) the receiver, placed under the cage, collected and converted data for (III) the Dataquest® A.R.T: PC-based system for the acquisition and analysis of data. After the start of the experiments, data were collected for 24 hours a day.

Surgical procedures

For anaesthesia, a mixture containing one part of Hypnorm® (0.315 mg/ml fentanyl and 10 mg/ml fluanisone; Janssen Pharmaceutica, Belgium), one part of Dormicum® (5 mg/ml midazolam; Roche, The Netherlands) and two parts of sterile water was injected subcutaneously (SC) in a volume of 0.2 ml per 100 g of body weight.

The ventral abdomen was shaved using an electric shaver and disinfected. The peritoneal cavity was accessed with a midline abdominal incision. The catheter was inserted 1.5 cm into the abdominal aorta, the artery was sealed using surgical glue, and the transmitter was placed inside the peritoneal space. The abdominal incision was closed with non-absorbable suture incorporating the implant suture rib with interrupted stitches and finally the skin incision was closed. After the surgical procedures, animals were tattooed on the external ear.

Analgesia and postoperative care

The operated rats were housed individually and kept in a warm environment for the first 24 h after the surgery. Post-operative analgesia included Temgesic® (Buprenorphine hydrochloride, Reckitt & Colman Products Ltd, UK) 0.1 mg/kg SC twice a day and Rimadyl® (Carprofen, Pfizer, Belgium) 5 mg/kg SC once a day for 72 h. Twenty four hours later the operated rats were regrouped with two cage mates. One week before the experiments the condition of instrumented rats was evaluated on the basis of locomotor activity and cardiovascular function (data not shown). Animals were allowed to recover after surgery for 11 to 14 days prior to the experiments.

Experimental Design

Experiments were carried out in sets of two using a crossover design with a resting period between the experimental sets (Table 1). For both sets of experiments, only rats bearing telemetry transmitters were subjected to IG-gavage. The experiments were carried out on Mondays, Tuesdays, Wednesdays and Thursdays, always at 15.00. Room cleaning was carried out at 10.00 every day, except on Sundays. Water bottles were changed on Mondays and Fridays during the room cleaning. The cages were changed on Fridays at 15.00. On each experimental day, the scientist and the technician entered the room quietly just prior to the procedure. The rats were taken from their cages, immobilized with the scruff method – *i.e.* the scruff of the neck was carefully grasped between the thumb and forefinger. Handling with scruff was chosen because it appears to be the only common method to which habituation occurs (*Batūraitė et al., 2005*).

Each IG-gavage took approximately 40 seconds, and thereafter each rat was held by the scruff in the hand up to two min total time. The same experienced technician carried out all procedures with all the rats. After the rats had been treated, the personnel left the room and the animals were otherwise left undisturbed until the next morning. Nobody entered the room during the recordings.

Set I – Comparison of probes. IG-gavage was done using stainless steel or teflon probes as is shown in Table 1. The probes (Fuchigami, Japan) had silicone tips and were 95 mm (steel) or 100 mm (teflon) long with external diameter 1.8 mm.

IG-gavage was carried out according to Bekow & Baumans (2003). The probe length, inserted into the oesophagus, was measured for each individual rat as the distance from the nose to the caudal end of xiphoid cartilage. In accordance with the manufacturer's instructions, the probe was moistened with water. The probe was introduced to the back of the mouth and both the rat and the probe were aligned along the same axis. The

Table 1. Study design.

Rat	Weeks 1-2	Week 3 Mon-Thu	Weeks 4-5	Week 6 Mon-Thu	Weeks 7-8	Week 9			
		Set I. Comparison of probes				Set II. Comparison of volumes (ml/kg) Mon Tue Wed Thu			
1	Operation & recovery	Steel	Resting period	Teflon	Resting period	2	4	6	8
2		Steel		Teflon		6	8	2	4
3		Teflon		Steel		2	4	6	8
4		Teflon		Steel		6	8	2	4
5		Steel		Teflon		4	6	8	2
6		Steel		Teflon		8	2	4	6
7		Teflon		Steel		4	6	8	2

pharyngeal reflex was elicited and then the probe was slid down the oesophagus to its pre-measured length. During these experiments no solutions were administered, and the probe was held in position for 5 seconds.

Set II – Comparison of volumes. This was carried out 17 days after the previous set of experiments. During this entire series of injections, the same type of stainless-steel probes with silicone tips as in *Set I* was used and, depending on the administered volume, plastic 1.0...5.0 ml disposable syringes were used. The IG-gavage was carried out as described previously, but different volumes of physiological saline were administered, as shown in the Table 1. In experiments into the effect of different administration volumes, food was removed at noon on the day of the experiment and the procedures were carried out at 15.00.

In addition, the cardiovascular changes induced by routine cage change were observed on three Fridays following IG-gavage series earlier that week. The cage change was performed by the same laboratory technician with the same methodology.

Statistics

Mean indices of cardiovascular function – blood pressure, heart rate – registered during 60 min

before the experiment were considered baseline for each rat. Individual changes from the baseline (*i.e.* mean of the previous 60 min) were calculated for each rat and data were subjected to further statistical analysis.

The data were analyzed using repeated measures analysis of variance (MANOVA) using probe, volume and day as factors. *Post-hoc* statistical analysis was made by contrast analysis. Statistical analysis was carried out using the SAS System. Statistical significance was set at $p < 0.05$.

Results

Effect of IG-gavage on cardiovascular parameters with different types of probes

IG-gavage induced a significant increase in diastolic and systolic blood pressure and heart rate, lasting for about 40 minutes. Further analysis demonstrated a significant effect of probe on diastolic blood pressure (Wilks' Lambda 0.061, F value 27.34 Num DF 20, Den DF 36, $Pr > F$ 0.0001), systolic blood pressure (Wilks' Lambda 0.046, F value 37.20 Num DF 20, Den DF 36, $Pr > F$ 0.0001) and heart rate (Wilks' Lambda 0.045, F value 37.83 Num DF 20, Den DF 36, $Pr > F$ 0.0001) during 40 min after IG-gavage. As can be seen from Figure 1, IG-gavage with a stainless steel probe induced significantly larger changes in cardiovascular function.

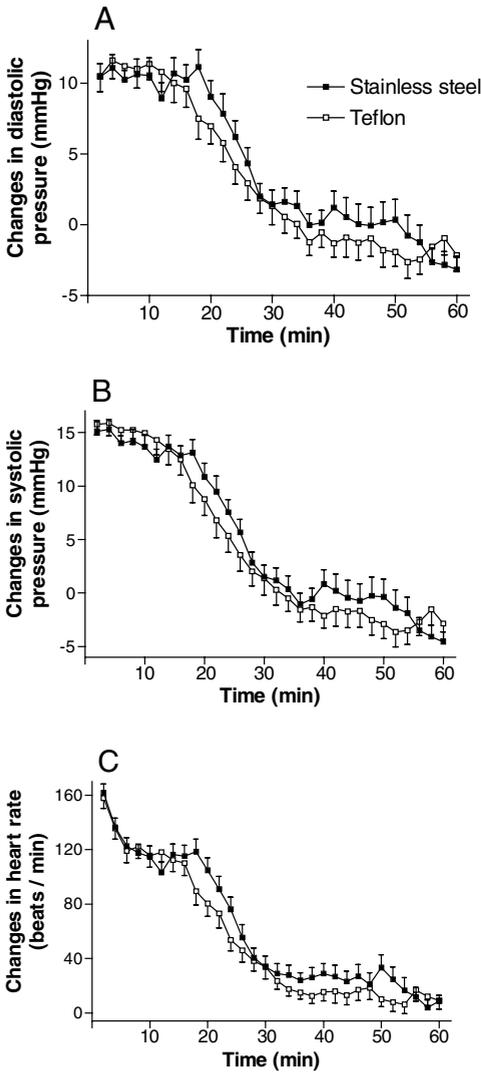


Figure 1. Effect of IG-gavage with stainless steel and teflon probes on changes of diastolic blood pressure (A), systolic blood pressure (B) and heart rate (C) from the baseline – *i.e.* mean indices of each parameter 60 min before the experiment. Data represents mean \pm SEM of 4 days from groups of 7 rats. Stainless steel probe had significantly stronger effect on diastolic blood pressure ($P < 0.05$), systolic blood pressure ($P < 0.05$) and heart rate ($P < 0.05$).

Habituation to IG-gavage

The effect of repeating the procedure is shown in Figure 2. There were significant effects of the day on diastolic blood pressure (Wilks' Lambda 0.062, F value 27.34 Num DF 20, Den DF 36, $Pr > F$ 0.0001), on systolic blood pressure (Wilks' Lambda 0.046, F value 37.20 Num DF 20, Den DF 36, $Pr > F$ 0.0001) and on heart rate (Wilks' Lambda 0.045, F value 37.83 Num DF 20, Den DF 36, $Pr > F$ 0.0001) during 40 min after IG gavage. As shown in Figure 2, changes due to repeated IG-gavage peaked on the third day, and then decreased back to initial or even lower values. In the teflon probe group, changes were lower on the fourth day as compared with the previous days, this being even clearer than noted in the stainless steel group.

Administration volume

MANOVA revealed a significant effect of volume on diastolic blood pressure (Wilks' Lambda 0.011, F value 34.80, Num DF 20, Den DF 8, $Pr > F$ 0.0001), systolic blood pressure (Wilks' Lambda 0.009, F value 23.65, Num DF 23, Den DF 5, $Pr > F$ 0.0012) and heart rate (Wilks' Lambda 0.006, F value 64.25 Num DF 20, Den DF 8, $Pr > F$ 0.0001). Surprisingly, volumes of 2 and 4 ml / kg body weight resulted in larger changes in cardiovascular function than volumes of 6 and 8 ml/kg, as shown on Figure 3.

Effect of week and probe type on cardiovascular response to cage change

The routine cage change on Fridays induced an increase in diastolic blood pressure, systolic blood pressure and heart rate, and the extent of the changes was comparable to the effects of IG-gavage (Figure 4). When we compared alterations after cage changes on weeks 2, 3 and 6 it emerged that the impacts on diastolic blood pressure ($F(2,1437) = 51.470, p = 0.000$), and systolic blood pressure ($F(2,1437) = 29.636, p = 0.000$) were significantly larger after the first change. There were no differences in cage change responses following either steel- or Teflon-probe gavage.

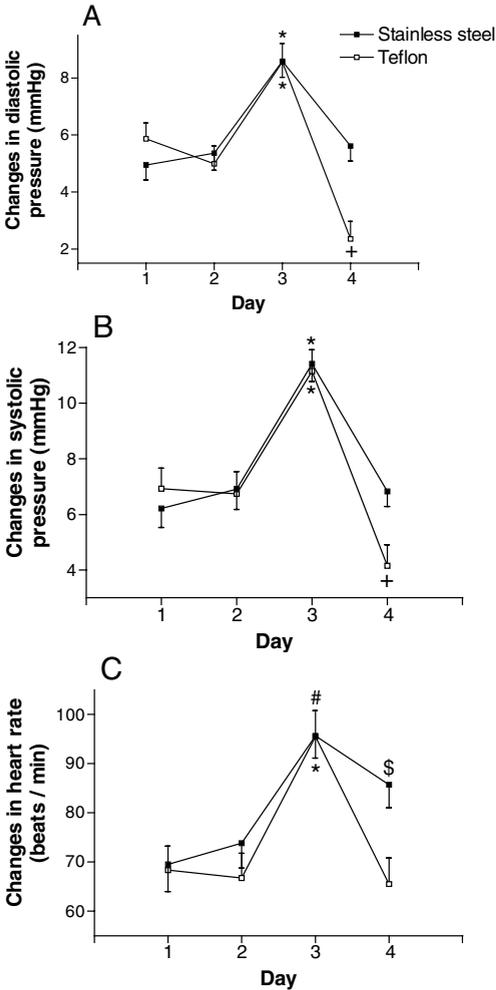


Figure 2. Habituation to IG-gavage with stainless steel and teflon probes. On this figure changes in diastolic blood pressure (A), systolic blood pressure (B) and heart rate (C) are shown. Data are presented as mean changes \pm SEM from the baseline of seven rats registered during 40 min.

* – $P < 0.01$ vs. Days 1,2,4 of the corresponding group (steel or Teflon);
 + – $P < 0.01$ vs. Days 1,2,3 of the corresponding group (steel or Teflon);
 # – $P < 0.01$ vs. Days 1,2 of the corresponding group (steel or Teflon);
 \$ – $P < 0.01$ vs. Day 1 of the corresponding group (steel or Teflon)

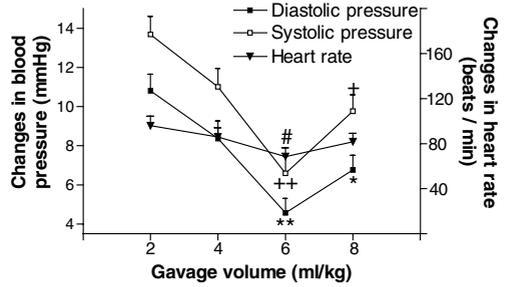


Figure 3. Changes in systolic and diastolic blood pressure (y_1) and heart rate (y_2) caused by the administration of different volumes via IG-gavage. On this figure change from the baseline (60 min before the procedure) following IG-gavage for 40 min is shown. Data presented are means \pm SEM from groups of 7 rats.

* – $P < 0.01$ vs. 2 ml/kg, ** – $P < 0.01$ vs. 2 ml/kg and 4 ml/kg (diastolic pressure);
 + – $P < 0.01$ vs. 2 ml/kg, ++ – $P < 0.01$ vs. 2 ml/kg and 4 ml/kg (systolic pressure);
 # – $P < 0.05$ vs. 2 ml/kg (heart rate)

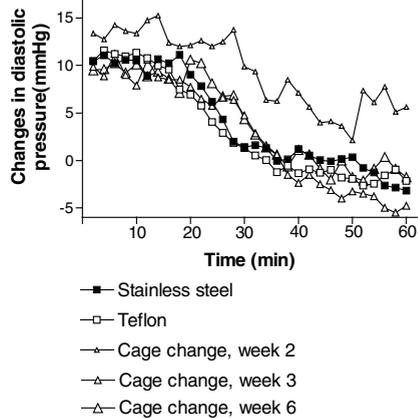


Figure 4. Comparison of the effects of IG and cage change on change in diastolic blood pressure. On this figure mean changes from the baseline registered during 60 min are shown. Data are means from groups of 7 rats. Changes following cage change on week 2 were significantly ($P < 0.05$) more pronounced than those following cage changes on week 3 and 6 or those evoked by IG-gavage.

Discussion

There should be two approaches to refining procedures on animals. The main emphasis thus far has been on addressing painful procedures and on methods to alleviate pain and distress. At the same time it is necessary to improve the overall welfare of laboratory animals. This latter approach represents small improvements for a large number of animals, while the number of animals exposed to painful procedures is far smaller. Both approaches are likely to yield considerable refinement. IG-gavage is a procedure carried out repeatedly on a large number of animals. In addition to avoiding morbidity and mortality due to IG-gavage (McIntyre, 2001; Murphy *et al.*, 2001; Rao *et al.*, 2001), there are ways in which this procedure could be refined. Cardiovascular telemetry appears to be a promising method to assess perception of discomfort and associated pain (Alban *et al.*, 2001) and it was utilized to identify improvements to the IG-gavage procedure.

In addition to animal welfare aspects, there are also scientific factors to be considered. Physiological, metabolic, endocrine and behavioural changes can be attributed to stress, and IG-gavaging procedures as such may interfere with the evaluation of novel drugs administered by this route.

Stiff probes made of steel are commonly used in laboratory rodents, though they would never be used in larger animals, such as rabbits, dogs or non-human primates. In this study IG-gavage with both probe types, steel and teflon, induced an increase in blood pressure and heart rate, which lasted for about 40 min. However, the material from which the probe is constructed is also a critical factor: thus the steel probe evoked a greater effect on the cardiovascular parameters. It has been shown that stress and fear in laboratory animals are accompanied by an increase in the heart rate and blood pressure (Morton & Hau, 2003; Van den Buuse *et al.*, 2001, 2002). Based on this finding, it is concluded that soft probes are preferable because they elicit less unpleasant feelings in the animals.

Rats have a strong gastroesophageal barrier, which consists of crural sling, which is part of diaphragm

and the lower oesophageal sphincter, located several centimeters distant along the intraabdominal oesophagus (Soto *et al.*, 1997). The oesophageal sphincter is a circular muscle, which contains muscle fibers inserting into gastric limiting ridge. When the sphincter contracts, it also pulls the sides of the limiting ridge together, thus hiding and tightly closing the esophageal opening (Montedonico *et al.*, 1999; McKirdy & Marshall, 2001; Botha, 1958). The strength of the three-step gastroesophageal barrier not only prevents reflux, but can also be thought to provide considerable resistance against the passing of a probe. Once in the oesophagus, a soft probe is more likely to be more correctly aligned and evoke less sensations, all of which may contribute to its lower propensity to evoke cardiovascular responses.

All soft probes are associated with probe breakage and this can lead to aspiration or simple misdirection of the administered substance. Although, the risk for breakage and the aspiration has been mentioned (Murphy *et al.*, 2001) there is very little hard data on their incidence. The success rate of uneventful IG-gavage is dependent on the experience of the technician and cooperation of the animal, perhaps achievable with habituation. Probe breakage can be prevented with the use of mouth gags, such as those proposed by Waynforth & Flecknell (1992) and these gags can be used with soft tubes. It remains to be studied whether soft probes in conjunction with such gags would elicit less dramatic cardiovascular responses than those evoked by steel probes.

All training about the use of animals emphasizes the need for habituation, *i.e.* activities aiming and increasing the familiarity of the animal to the procedure and to the surroundings. In standard tests for anxiety, if the animal is accustomed to the experimenters this will improve consistency of the results (Van Driel & Talling, 2004), and even short habituation to scruff handling has been shown to decrease the duration of cardiovascular response in rats (Batūraitė *et al.*, 2005).

Repeated IG-gavage induced different changes in stainless steel and teflon groups. While in the teflon

probe group, changes were less on the fourth day as compared with previous days, in the stainless steel group the extent of the habituation was not observed (Figure 2). Overall, it appears that response increased until the third day of repeated administration, and then decreased considerably. It is not known what would have happened with a more prolonged schedule or whether a weekend break would have broken the habituation.

Perhaps the most interesting and intriguing finding of this study was that response to different IG-gavage volumes was not linear as might have been anticipated. Since we believe that these cardiovascular parameters do reflect the level of stress, it appears that IG-gavage volumes of 6 and 8 ml per kg of body weight were less stress-provoking than gavage volumes of 2 and 4 ml/kg. Thus, it appears that less is not necessarily better, but rather that there is a window of optimal administration volumes.

It has been suggested that if part of the administered dose passes into the duodenum, the discomfort may be less than if the compound is retained in the stomach. In unanesthetized rats, larger volumes (32–40 ml/kg) via intragastric gavage induce spontaneous release into the duodenum (Alban *et al.*, 2001). There is little hard data, in fact much of it contradictory, on the gastric volume of rats, and even these data are based on rather variable administration methods. For example, in the study of Liao *et al.* (2005), using 300 g rats, 15 ml were placed *in vitro* into the isolated rat stomach, while Graça *et al.* (2000) concluded that the mean gastric volume of anesthetized rats, weighing 250–300 g was 2.88 ± 0.13 ml. Since we used rats of approximately the same weight, 310–390 g, it is possible that the larger volumes used in our study 6 and 8 ml / kg, did permit spontaneous release of the administered vehicle into the duodenum.

The results of this study are different from those of Bonnichsen *et al.* (2005) who claimed that administration volume was of minor importance in the stress evoked by IG-gavage in rats. The difference may not be large, but it does mean that refinement can be

achieved by optimizing the volume. While Bonnichsen *et al.* used a large range (0, 4, 10 and 40 ml / kg body weight) this study did not proceed to extremes, but utilized 2, 4, 6 and 8 ml / kg body weight instead. In short, it appears that the sensitivity of rats to volume is higher in low IG-gavage volumes. Another tentative explanation for this discrepancy is different gavage solutions. While Bonnichsen *et al.* (2005) used BaSO₄ solution, saline was used in this study. Since the viscosity of the solution has been shown to have an effect on the stress level (Brown *et al.*, 2000), it may be that rats are more sensitive to low-viscosity solutions. However, the volume-response curve of IG-gavage will need further study. The choice of administration volume is also influenced by other factors. Brown *et al.* (2000) demonstrated that very small volumes of low-viscosity vehicles are associated with increased risk of volume retention in the oesophagus and aspiration into the respiratory tract.

It is also possible that changes in cardiovascular function, caused by the administration of different volumes, could be mediated via the tension applied to the gastric wall. Römer *et al.* (2005) reported that gastric distension induced a decrease in mean arterial pressure, the magnitude of which was related to the intragastric pressure applied. The higher volumes used in this study could indeed have caused an elevation in the tension of the gastric wall.

Even though the observed differences between teflon and stainless steel probes are statistically significant, the physiological significance has to be addressed as well. Krohn *et al.* (2003) have suggested that a change of 5% or more in cardiovascular parameters can be considered to have practical welfare implications. Hence, the results of this study on probe types and habituation can be considered as borderline cases, but valid for refinement comparisons, while those on administration volumes have direct welfare value.

Comparison of the cardiovascular changes revealed that IG-gavage causes effects of the same magnitude and duration as routine cage change. The time-related decrease in response to cage change cannot

be attributed to habituation alone, but rather to the combination of procedures and cage change (Figure 4). The significant effect of cage change on cardiovascular function is in line with previous data in the literature (Duke *et al.*, 2001; Sharp *et al.*, 2003a, 2003b) indicating that common husbandry procedures – *i.e.* cage change, handling and weighing. – do alter the cardiovascular function of rats.

In conclusion, IG-gavage with a soft teflon probe in rats appears to be less stressful. According to changes in the blood pressure, habituation develops to IG-gavage with soft probes, but not with stainless steel probes. Therefore, when possible, soft probes should be preferred. Administration volumes in the range of 6-8 ml/kg seem advisable, and additionally allow better dose precision than smaller volumes. Finally the amount of stress attributable to IG-gavage in rats is comparable to routine cage change, and hence may be considered not as a major stress.

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