

Effects of Anaesthesia on Insulin Sensitivity and Metabolic Parameters in Wistar Rats

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Abstract. We compared the effects of two different anaesthetics, sodium pentobarbital (65 mg/kg) and ketamine (30 mg/kg) plus xylazine (4 mg/kg) (KX) on insulin sensitivity, fasting glycaemia, insulinaemia and free fatty acids (FFA). Four groups of Wistar rats were used: KX group (n=6), pentobarbital group (n=6), high-sucrose diet group (n=6) and the conscious group (n=6). The insulin tolerance test (ITT) was used to measure insulin sensitivity, and metabolic biomarkers were determined using commercial kits. Pentobarbital did not alter plasma insulin, glucose, FFA or the ITT results compared to conscious animals. In contrast, KX anaesthesia induced hyperglycaemia, increased serum FFA and altered the ITT results compared to the conscious animal group. Moreover, under pentobarbital anaesthesia, the ITT proved to be a suitable method to detect insulin resistance in an animal model of diet-induced insulin resistance. We concluded that sodium pentobarbital anaesthesia should be used in metabolic studies since it does not interfere with plasma glucose, insulin, FFA or insulin sensitivity quantification in Wistar rats.

Insulin resistance is a central feature in the pathophysiology of diabetes, metabolic syndrome and obesity. Quantifying insulin resistance in animals by means of a standardized procedure has become a prominent issue. Particularly in laboratory animals, the optimal choice and employment of a specific method has to take into account animal welfare, namely the use of anaesthetics during the procedures to avoid pain and distress. Sodium pentobarbital is a long-acting barbiturate commonly used to induce general anaesthesia in

animals. It produces hypnosis, although it has no analgesic or skeletal muscle relaxation properties, and when administered intraperitoneally, it induces a state of surgical anaesthesia. Ketamine is a dissociative anaesthetic that provides light surgical anaesthesia but poor muscle relaxation. It is most effective when combined with a sedative drug such as the alpha-2 adrenergic agonist, xylazine. Sodium pentobarbital and the combination of ketamine and xylazine have been widely used in metabolic studies; nevertheless, it is still not clear how insulin sensitivity is affected by anaesthesia. In order to evaluate the influence of anaesthesia on insulin sensitivity in Wistar rats, we conducted a study whereby insulin sensitivity was comparatively evaluated in conscious animals and animals anaesthetized with two different strategies. We hypothesized that anaesthesia with sodium pentobarbital at a dose of 65 mg/kg *i.p.* minimizes interference with the measured outcome, providing a suitable means of anaesthesia for evaluation of insulin sensitivity. Secondly, we tested if the insulin tolerance test (ITT) is a suitable tool to distinguish insulin-sensitive from insulin-resistant sucrose-fed rats, under pentobarbital anaesthesia.

Animals. Experiments were carried out in male SPF outbred Wistar rats, obtained from the animal house of the Faculty of Medicine of Nova University, Lisbon, Portugal. After weaning, they were maintained in rooms under temperature control at 22±2°C and relative humidity of 55±10%. Lights were on a 12-h light-dark cycle (08:00-20:00 h). The rats were housed in social groups of three, randomly distributed among plastic cages with 1195 cm² of floor area and 14 cm of height (Tecniplast, Buguggiate, Italy). Each cage was provided with standard corn cob litter (Probiológica, Lisbon, Portugal). Rats were fed *ad libitum* with rodent pellets RM1, SDS diets[®] (obtained from Probiológica, Lisbon, Portugal), except for the day before the experiment, when they were submitted to an overnight fast but with free access to water. All animals drank deionised water. Cages were changed once a week (1). Animals were tested between 9 and 11 weeks of age. At the end of the experiment,

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the anaesthetized animals were euthanized with an overdose of intracardiac sodium pentobarbital and the conscious rats were euthanized with 70% carbon dioxide in an euthanasia chamber. All procedures were carried out in compliance with the principles expressed in the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (86/609/CEE). The protocols were approved by the Animal Care and Use Committee of the Faculty of Medicine of Nova University of Lisbon.

Materials and Methods

Experimental design. A preliminary study was carried out to evaluate the effect of the different anaesthetics on blood glucose levels and to choose the dose of insulin to be used in the ITT. We tested the two insulin doses most frequently used in ITT in clinical and research settings (2). We used pentobarbital-anaesthetized animals in the dose-response study due to the hyperglycaemic response induced by ketamine-xylazine anaesthesia, which may interfere with insulin sensitivity determination. The study had two stages. The first was designed to test the hypothesis that the action of insulin was influenced by different types of anaesthetics, and aimed to identify the most suitable anaesthetic when performing an ITT in rats. To do so, an ITT was performed in conscious animals and in animals anaesthetized with two different drugs: sodium pentobarbital at 65 mg/kg and a combination of ketamine at 30 mg/kg and xylazine at 4 mg/kg. The second stage of the study tested if the ITT performed under the effect of the best-suited anaesthetic, determined in the first part of the study, was a suitable tool to distinguish between insulin actions in insulin-sensitive and insulin-resistant groups of animals.

Effect of sodium pentobarbital and KX on insulin sensitivity. Eighteen rats weighting 251 ± 9.7 g were fasted overnight. The animals were randomly divided into three groups. In the first group (here termed the KX group), animals were anaesthetized with ketamine at 30 mg/kg plus xylazine at 4 mg/kg; in the second group the animals were anaesthetized with sodium pentobarbital at 65 mg/kg. A third group of conscious animals was used as control; these animals were placed in a rat restrainer during the experimental procedure. The animals from the KX and sodium pentobarbital groups were always restrained and anaesthetized by the same researchers. The anaesthetic was injected *i.p.* with a short 25-gauge needle, which was inserted at an angle of 45° to the abdominal wall in the lower left quadrant (maximum injected volume: 0.5 ml); afterwards the animal was placed in a cage with bedding material, alone, until the loss of the righting reflex. The rat was then transferred to a heating pad to maintain body temperature at $37.5 \pm 0.5^\circ\text{C}$ during the whole experiment. Temperature was monitored with a rectal thermometer.

Effect of sodium pentobarbital anaesthesia on insulin sensitivity in rats submitted to a high-sucrose diet. Previous reports have shown that feeding of a 35% sucrose solution to Wistar rats induces insulin resistance without overt hyperglycaemia (3, 4). In a second set of experiments, 12 Wistar rats (257 ± 8.3 g) were randomly divided into two groups. The first group drank regular deionised water (here termed the control group) and the second group drank a 35% sucrose solution (here termed the S group) for a period of four weeks. Body weight and water intake were evaluated daily in both groups. The animals were

fasted overnight with free access to water and anaesthetized *i.p.* with sodium pentobarbital at 65 mg/kg as described above; body temperature was recorded and controlled as described above.

The ITT. The ITT was used to evaluate insulin sensitivity (2). It consisted in the administration of an intravenous insulin bolus of 0.1 U/kg body weight, after an overnight fast, followed by the measure of the decline in plasma glucose concentration over 15 min. Insulin sensitivity was evaluated by the K_{ITT} parameter, *i.e.* the slope of the linear decline in glycaemia multiplied by 100. ITT was performed in both anaesthetized and conscious rats. In the anaesthetized group, after confirmation of anaesthesia, tail tipping was used to collect drops of blood. In the conscious group, the same method was used to collect blood samples. Blood glucose levels were measured using a Glucometer Precision X-Ceed and test strips (Abbott Diabetes Care, Lisbon, Portugal). When the baseline glucose level measured was stable, an insulin bolus of 0.1 U/kg body weight, dissolved in saline to perform a 0.1 ml fixed volume, was administered in the tail vein and the blood glucose was measured every 2 min. At minute 16, the test stopped. The slope of blood glucose decay was calculated by linear regression and the constant rate for glucose disposal (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$. Glucose half-time ($t_{1/2}$) was obtained from the slope of the least square analysis of plasma glucose concentrations during the linear decay phase. The homeostatic model assessment (HOMA index) was also calculated as $I_0(\text{fasting insulinaemia}) * G_0 (\text{fasting glycaemia}) / 22.5$ (5).

Measurement of insulinaemia and free fatty acids (FFA). Plasma and serum were collected after heart puncture to EDTA pre-coated tubes and to eppendorfs, respectively and kept on ice. Plasma samples were centrifuged at $3,000 \times g$ for 10 min (4°C) and serum samples were centrifuged in a microfuge (Eppendorf, Madrid, Spain) at $12,000 \times g$ during 10 min and stored at -80°C in an ultra-low freezer (Heraeus, Madrid, Spain). Plasma and serum were used for quantification of insulinaemia and FFA with an ELISA kit (Mercodia Ultrasensitive Rat insulin ELISA kit; Mercodia AB, Uppsala, Sweden) and a colorimetric assay (Zenbio, Denver, NC, USA), respectively.

Chemicals. The chemicals came from the following sources: ketamine chloridrate (Imalgene 1000) from Merial, Lyon, France; xylazine chlorhydrate (Rompum® 2%) from Bayer Portugal SA, Carnaxide, Portugal; pentobarbital (Eutasil, 20 mg/kg) from CEVA, Sante-Animale, Portugal; insulin (Humulin Regular) from Lilly, Oeiras, Portugal. Standard physiological saline 0.9% was from B-Braun, Queluz de Baixo, Portugal.

Data analysis. Data were evaluated using the Graph Pad Prism Software, version 4 (GraphPad Software Inc., San Diego, CA, USA) and are presented as the mean \pm SEM. The significance of the differences between the means was calculated by one and two-way analysis of variance (ANOVA) with Dunnett's and Bonferroni multiple comparative tests, respectively. Potential relationships between fasting glucose levels and K_{ITT} were explored using the Pearson's correlation coefficient. Differences were considered significant at the $p \leq 0.05$ level.

Results

Effects of sodium pentobarbital and KX on blood glucose levels. Figure 1 represents the changes in blood glucose observed immediately after *i.p.* administration of the

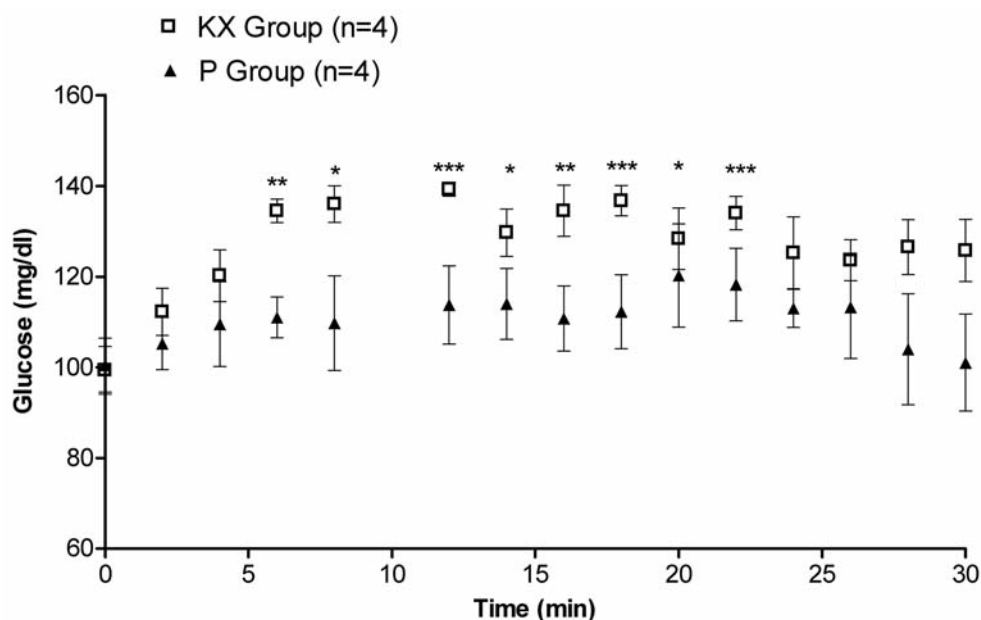


Figure 1. Effect of anaesthesia with ketamine/xylazine (KX: 30 mg/kg/4 mg/kg) and sodium pentobarbital (P: 65 mg/kg) on blood glucose levels over a timeline of 30 minutes. Each point represents the mean \pm SEM of four animals. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, after performance of two-way ANOVA with Bonferroni multiple comparison test.

anaesthetics. We observed that the combination of KX ($n=4$) induced a significant rise in blood glucose values 6 minutes after induction of anaesthesia ($p < 0.001$). This hyperglycaemic effect was sustained until minute 22. Administration of sodium pentobarbital ($n=4$) did not change the glucose level for a period up to 30 minutes after induction of anaesthesia.

Insulin dose- K_{ITT} response relationship in pentobarbital anaesthetized animals. The K_{ITT} obtained with an intravenous insulin dose of 50 mU/kg was 2.26 ± 0.40 %glucose/min. The dose of 100 mU/kg led to a K_{ITT} of 4.65 ± 0.57 % glucose/min ($p < 0.01$). We chose to use the dose of 100 mU/kg in the subsequent studies because the response is higher, which allows for characterization of insulin-sensitive and insulin-resistant animals.

Evaluation of the effect of different anaesthetics on insulin sensitivity and metabolic parameters. Based on the preliminary findings plotted in Figure 1, ITTs were started 10 minutes after the induction of anaesthesia in order to perform the ITT during the time period of steady blood glucose values. Figure 2 illustrates representative results of ITTs obtained in one experiment of the conscious control group (panel A), one experiment of the pentobarbital group (panel B) and one experiment of the KX group (panel C); K_{ITT} values obtained in the cases illustrated were 3.01 %glucose/min (Figure 2A), 4.21 %glucose/min (Figure 2B) and 5.17 %glucose/min (Figure 2C). The mean K_{ITT} was 4.02 ± 0.68 %glucose/min in the conscious

group, 4.21 ± 0.23 %glucose/min in the pentobarbital group and 5.93 ± 0.38 %glucose/min in the KX group, which was significantly different from the K_{ITT} in conscious rats ($p < 0.01$), indicating that the KX group exhibited higher insulin sensitivity. The blood glucose levels at the beginning of the test differed significantly between groups, being highest (137.7 ± 10.6 mg/dl) in the KX group ($p < 0.001$ vs. conscious group, Table I). Fasting insulin levels were not significantly different in the conscious rats compared to the pentobarbital group or the KX group (Table I). KX, but not pentobarbital, significantly increased plasma FFA in the KX group ($p < 0.01$ vs. conscious group, Table I). The HOMA index, which is inversely correlated with insulin sensitivity was not significantly different in conscious and pentobarbital-anesthetized rats, at 2.68 ± 1.05 vs. 3.67 ± 1.19 , respectively; however, in the KX group, the HOMA index was 14.03 ± 3.16 , which was significantly different from that of the conscious group ($p < 0.01$).

Effect of sodium pentobarbital anaesthesia on insulin sensitivity in rats submitted to a high-sucrose diet. The average daily liquid intake was 28.4 ± 1.22 ml/day in the control group and 26.8 ± 1.06 ml/day in the sucrose group. Body weight was not significantly different between groups: 261 ± 14.9 g in control group and 253 ± 8.7 g in the sucrose group. All the experiments were performed under sodium pentobarbital (65 mg/kg) anaesthesia based on the results described above. The plots in Figure 3A and 3B illustrate the ITT performed in an animal representative of the control group and of the

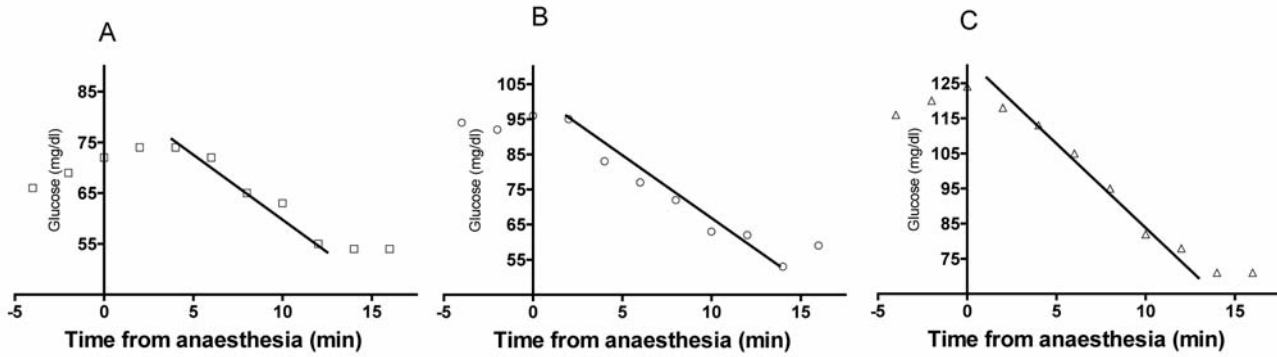


Figure 2. Representative experiments of the effect of administration of 100 mU/kg insulin on blood glucose levels in a conscious animal (A), an animal anaesthetized with sodium pentobarbital at 65 mg/kg (B) and an animal anesthetized with ketamine at 30 mg/kg plus xylazine at 4 mg/kg (C).

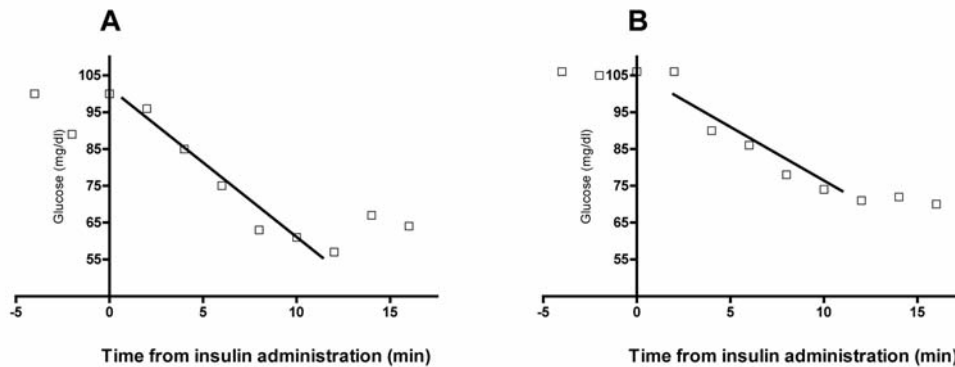


Figure 3. Representative experiments of the effect of administration of 100 mU/kg insulin on blood glucose levels in rats that drank regular deionized water (A) and rats that drank a 35% sucrose solution for four weeks (B). All the animals were anaesthetized with sodium pentobarbital at 65 mg/kg.

sucrose-fed group, respectively. K_{ITT} values were 5.56 %glucose/min and 3.71 %glucose/min, respectively. The mean K_{ITT} was 4.46 ± 0.39 %glucose/min in the control group and 2.22 ± 0.34 %glucose/min in the sucrose group ($p < 0.001$). As shown in Table I, basal glucose level was 133.5 ± 1.78 mg/dl in sucrose-fed animals, which was significantly higher than that in control animals ($p < 0.001$ compared with pentobarbital group). Both fasting insulin and serum FFA levels were significantly higher in the sucrose-fed animals ($p < 0.001$ and $p < 0.01$ compared with pentobarbital group, Table I).

Study of the relation between glucose level at t=0 and insulin sensitivity. To test if the blood glucose concentration at the beginning of the test influenced insulin sensitivity, we characterized the relation between blood glucose level at t=0 and the value of K_{ITT} . All the experiments were pooled and the correlation between K_{ITT} and plasma glucose was tested. There was a significant correlation between K_{ITT} and plasma glucose at t=0 (Pearson correlation coefficient was $r = 0.4977$ and $p = 0.0051$, Figure 4).

Table I. Effect of anaesthesia and sucrose treatment on plasma glucose, insulin and serum-free fatty acids (FFA).

	Glucose (mg/dl)	Insulin (μ g/L)	FFA (μ M)
Conscious (n=6)	68.0 ± 4.66	0.64 ± 0.31	274.1 ± 9.1
Pentobarbital (n=6)	89.7 ± 4.03	0.62 ± 0.21	342.2 ± 31.5
Ketamine/Xylazine (n=6)	$137.7 \pm 10.6^{***}$	0.84 ± 0.38	$514.6 \pm 82.1^{***}$
Sucrose-fed (n=6)	$133.5 \pm 1.78^{###}$	$5.5 \pm 0.30^{###}$	$891.1 \pm 93.3^{###}$

*** $p < 0.001$ vs. conscious animals, one-way ANOVA with Bonferroni multiple comparison test between conscious, pentobarbital and KX groups. ### $p < 0.001$ vs. pentobarbital animals, Student's *t*-test.

Discussion

In the present study we analysed the effects of pentobarbital and KX anaesthesia on metabolic homeostasis and insulin sensitivity. Physiological variables are influenced by drugs often used in studies with laboratory animals, namely

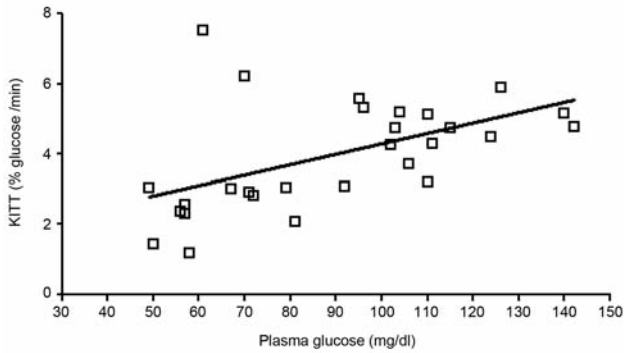


Figure 4. Correlation between insulin sensitivity (K_{ITT}) and basal plasma glucose at the beginning of the ITT ($t=0$). Data represent all the individual samples obtained from 8-14 rats in the four test groups. The Pearson correlation coefficient was $r=0.4977$ and $p=0.0051$.

anaesthetic agents. This specific class of drug is a valuable tool for alleviating pain and distress in animals based on welfare, ethical, but also for scientific reasons, since it is known that discomfort can affect a large number of organs and their physiological responses. Nevertheless the anaesthetics themselves can interfere with the experimental protocol. Therefore, in order to improve the reliability of the model being used, the choice of the anaesthetic, or combination of anaesthetics, has to be made according to the experimental design and the variables and outcomes of interest. We concluded that while pentobarbital did not change any of the metabolic parameters tested compared to conscious animals, the combination of ketamine and xylazine caused increased K_{ITT} , fasting hyperglycaemia, increased HOMA index and elevated serum FFA. We also concluded that the ITT carried out under sodium pentobarbital anaesthesia is a suitable tool for evaluating insulin resistance in Wistar rats, representing a methodological alternative to alleviate discomfort in terminal experiments. With the doses used we did not observe mortality under pentobarbital or KX anaesthesia. The technique chosen to evaluate insulin sensitivity was the ITT, one of the first methods developed to assess insulin sensitivity *in vivo* and which is widely used in clinical and basic research settings (2, 6-10). This method requires, in the worst case scenario, 20 blood drops from the tail tip collected for 45 minutes. Our results suggest that performing the ITT in pentobarbital-anaesthetized rats avoids the stress induced by the puncture of the tail vein for insulin administration and does not require restraining of the animal for 30 minutes for collection of blood samples, avoiding at the same time interferences in glucose homeostasis induced by acute stress responses. Whenever recovery of anaesthesia is included in an experimental protocol, the use of sodium pentobarbital to perform an ITT must be re-evaluated since there is also distress associated with recuperation from the

anaesthesia. However, most experiments in the endocrine and metabolic fields are terminal experiments that require tissue sample collection and in these cases the anaesthetized approach becomes adequate in terms of refinement. Our results are consistent with the findings of Saha *et al.* who observed that KX (100 mg/kg ketamine and 10 mg/kg xylazine) caused acute hyperglycaemia in fed Sprague-Dawley rats, whereas sodium pentobarbital at 60 mg/kg did not (11). Other authors have also observed that KX caused glucose intolerance, in which the glucose concentrations were elevated for 5 hours after intraperitoneal administration of 2 g glucose/kg bw 15 minutes after induction of anaesthesia (12). Under our experimental conditions, we also observed a hyperglycemic effect of KX despite the dose used in our study being smaller than the used by Saha *et al.* Intraperitoneal sodium pentobarbital anaesthesia has been shown by other groups to induce transient hyperglycaemia in Wistar rats (13, 14), while in other studies, the hyperglycaemic response was not observed (15, 16); these disparities may be explained by the different strains used in the studies. Herein, to our knowledge, we show for the first time that sodium pentobarbital anaesthesia does not interfere with insulin sensitivity quantification through the ITT technique, since there were no significant differences in K_{ITT} values for the conscious and pentobarbital-treated groups. The increase in K_{ITT} observed in the KX group was probably due to the hyperglycaemic effect induced by the combination of drugs, which made more glucose available for uptake during the ITT and increased K_{ITT} . This hypothesis is supported by the results shown in Figure 4, which show that K_{ITT} is correlated with the blood glucose values at the beginning of the ITT. Besides the striking changes in K_{ITT} , the animals anaesthetized with KX also had a significantly higher HOMA index and serum FFA level. These results confirm that KX anaesthesia modifies glucose homeostasis and also lipid homeostasis, making it inappropriate for use in metabolic studies in animals. We also tested the ability of the ITT to diagnose insulin resistance in a pathological animal model when performed under sodium pentobarbital anaesthesia. We observed that administration of a 35% sucrose solution reduced K_{ITT} and therefore insulin resistance, as previously described and detected by other insulin sensitivity evaluation methods (3). In humans, normal K_{ITT} (indicative of normal insulin sensitivity) is considered to be >2 %glucose/min and values <1.5 %glucose/min are considered indicative of insulin resistance (2). In sodium pentobarbital-anaesthetized rats, such a range of values does not apply, since the control K_{ITT} was 5.2 ± 0.45 %glucose/min and the K_{ITT} in the insulin-resistant group was 2.3 ± 0.39 %glucose/min. Although several groups have performed ITT in conscious animals in order to avoid the interference of anaesthetics (17-19), we conclude that under sodium pentobarbital anaesthesia the ITT represents a suitable, reliable and easy method to evaluating insulin

sensitivity in Wistar rats. In contrast for other methods of calculating the insulin sensitivity index, such as the oral glucose tolerance test (OGTT), where it has been shown that sodium pentobarbital reduces insulin sensitivity (13, 20, 21), the ITT can and should be performed under sodium pentobarbital anaesthesia, since the obtained KITT values do not significantly differ from those of conscious animals. Altogether, the results suggest that the ITT in sodium pentobarbital-anaesthetized animals is simple to perform, minimizes discomfort and avoids stress responses, which can shift insulin responsiveness. It is a suitable method for evaluating insulin sensitivity and should be performed as described in terminal experiments. The use of KX should be avoided due to its pronounced hyperglycaemic effect and dyslipidaemic effect. Moreover, when performed as described herein, the ITT distinguishes insulin-sensitive from insulin-resistant animals.

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