Anesthetic technique does not affect the performance of a rabbit model of arterial cyclic flow reductions: a pilot study

[La technique anesthésique n'affecte pas la performance d'un modèle de réductions cycliques de flot chez le lapin : une étude pilote]

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Purpose: Pentobarbital anesthesia is, typically, used in an experimental model of cyclic flow reductions (CFR) in rabbits. Our initial observations, using a more complete and effective isoflurane-based anesthetic technique, failed to reproduce findings reported previously. Consequently, we compared the effects of these two anesthetic techniques in the model.

Methods: A modified Folts' model of carotid artery lesion and stenosis was used. Twelve rabbits completed the experimental protocol: five in the pentobarbital group (P) and seven in the isoflurane group (I). The carotid artery was exposed and flow was reduced by application of a clamp. A standardized injury was performed by cross clamping the artery with a needle forceps and this produced CFR. The number of CFR and the duration of their occurrence were noted. The incidence of thrombosis was compared in each group as well as hemodynamic, hematologic and bleeding time values.

Results: The hematocrit value, platelet count and bleeding time were similar in both groups. The median number and range of CFR [group P: 9 (4–16) ; group I: 9 (5–14)] and the time span of effective CFR formation (group P: 39 ± 17 ; group I: 38 ± 25 min) were comparable in both groups. The incidence of complete thrombosis of the carotid artery was similar in both groups.

Conclusions: The stability of the model is of short duration, but the occurrence of CFR is not affected by the type of anesthesia. Our findings suggest that the ideal duration of the experimental protocol should be between 30 and 45 min in order to maximize the number of animals still developing CFR.

Objectif : Le pentobarbital est l'anesthésiant type utilisé dans un modèle expérimental de réductions cycliques de flot (CFR) chez les lapins. Nos observations initiales, en utilisant une technique anesthésique basée sur l'isoflurane, n'ont pas reproduit les observations rapportées précédemment. Par conséquent, nous avons comparé les effets de ces deux techniques anesthésiques chez le lapin.

Méthode : Un modèle de Folts modifié de lésion de l'artère carotide et de sténose a été utilisé. Douze lapins ont pris part au protocole expérimental : cinq dans le groupe pentobarbital (P), et sept dans le groupe isoflurane (I). L'artère carotide a été exposée et le débit réduit par une clamp. Une lésion standardisée a été faite par clamping de l'artère carotide avec un forceps fin, ce qui a provoqué des CFR. Le nombre de CFR ainsi que la durée pendant laquelle ces réductions avaient lieu ont été enregistrés. L'incidence de thrombose a été comparée dans chaque groupe, tout comme les valeurs hémodynamiques, hématologiques, et le temps de saignement.

Résultats : La valeur de l'hématocrite, la numération plaquettaire et le temps de saignement ont été similaires dans les deux groupes. Le nombre moyen et la portée des CFR [groupe P : 9 (4–16) ; groupe I : 9 (5–14)] ainsi que la durée pendant laquelle les CFR avaient lieu (groupe P : 39 ± 17 ; groupe I : 38 ± 25 min) ont donné des résultats comparables dans les deux groupes. L'incidence de thrombose complète de l'artère carotide a été similaire dans les deux groupes.

Conclusion : La stabilité du modèle est de courte durée, mais le nombre de CFR n'est pas affecté par le type d'anesthésie. Nos résultats suggèrent que la durée idéale du protocole expérimental devrait être de 30 à 45 min afin de maximiser le nombre d'animaux développant des CFR de manière soutenue.

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WITH a normally functioning hemostatic system, blood flow within an injured blood vessel is usually maintained. This implies a delicate balance between clot formation and lysis, two mechanisms that are activated rapidly and designed to be effective locally. Numerous physiological, pathological and pharmacological factors have the potential to modify normal blood hemostasis.

Folts' model of coronary thrombosis was developed in 1974 to study platelet aggregation and thrombosis in damaged and stenotic coronary arteries.^{1,2} In this model, endothelial and medial damages combined with a fixed artery stenosis produce periodic acute platelet thrombosis and cyclic flow reductions (CFR). Cyclic flow reductions are characterized by a progressive reduction of blood flow, followed by a sudden return to the pre-reduction level (Figure 1). This model, initially described in dogs, has been adapted to other animal species and for other vascular territories.^{3,4}

In cooperation with an expert in the field (Prof. Charles Marc Samama, Paris, France),³⁻⁹ we developed the model of arterial CFR in rabbits at the Centre Hospitalier de l'Université de Montréal (CHUM). In Paris, pentobarbital anesthesia is used for the entire procedure but this anesthetic technique was not acceptable to the veterinarian in charge of the animal laboratory at the CHUM. Barbiturates are poor analgesics and, when used alone, the dose required to produce surgical anesthesia is very close to the lethal dose.¹⁰ Consequently, we elected to employ a more effective and complete anesthetic technique.

During the development of our model in rabbits using isoflurane as the main anesthetic agent, we noted that the time span of effective CFR formation appeared to be no more than 45 to 60 min. Despite all our efforts to reproduce a well known model, our initial observations were contrary to published reports where the model, using pentobarbital anesthesia, is purported to be stable for prolonged periods of time.^{1,2} This led us to believe that the anesthetic technique might be responsible for the discrepancy between our preliminary observations and those published in the literature. Consequently, we compared the effects of two anesthesia protocols on the production of CFR using Folts' model in rabbits. We hypothesized that, because of its vasodilatory properties combined with a possible platelet inhibiting activity, anesthesia with isoflurane would produce less CFR and that CFR would be sustained over a shorter time span than during anesthesia with pentobarbital.

FIGURE 1 Cyclic flow reduction (CFR). A typical CFR occurring during a period of stable blood pressure and heart rate. Instantaneous carotid artery blood flow and mean carotid artery blood flow increase suddenly after a gradual decrease. Tracing from our laboratory.

Material and methods

The study was approved by our institution's animal protection committee. All animals were treated in accordance with the Guidelines of the Canadian Council on Animal Care. Twenty male New Zealand rabbits (Charles River Canada Inc., St-Constant, QC, Canada) weighing 2.9 ± 0.2 kg were divided in two groups according to the anesthesia protocol used for maintenance: isoflurane (group I) and pentobarbital (group P).

In all animals, anesthesia was induced with propofol ($15 \text{ mg}\cdot\text{kg}^{-1} \text{ iv}$) (Astra Zeneca Canada Inc., Mississauga, ON, Canada). The trachea was intubated with a size 3 Cole endotracheal tube and the lungs ventilated with a tidal volume of 40 mL at a rate of 40 breaths $\cdot\text{min}^{-1}$ ($F_{\text{I}}\text{O}_2 = 1.0$). Muscle relaxation was ensured with pancuronium bromide (Sabex Inc., QC, Canada) at a dose of $1 \text{ mg}\cdot\text{kg}^{-1} \text{ iv}$. Oxygenation was monitored by pulse oximetry, ventilation was monitored quantitatively with a capnograph and the end-tidal CO_2 was maintained between 35 and 45 mmHg. Body temperature was kept normal at 38.5°C with a warming blanket and hot packs. A 24-G femoral artery catheter was inserted for blood sampling and continuous arterial blood pressure recording.

In group P ($n = 9$), anesthesia was maintained with an infusion of pentobarbital $30 \text{ mg}\cdot\text{hr}^{-1} \text{ iv}$ (MTC Pharmaceuticals, Cambridge, ON, Canada) and fentanyl citrate $18.75 \text{ }\mu\text{g}\cdot\text{hr}^{-1}$ (Sabex Inc., QC, Canada) for a total volume of $6 \text{ mL}\cdot\text{hr}^{-1}$, adjusted to maintain a systolic blood pressure of 90–100 mmHg. Additional boluses of pentobarbital (15.5 mg) and fentanyl (7 μg) were given before skin incisions. In group I ($n = 11$), anesthesia was maintained with isoflurane (Baxter Corporation, Toronto, ON, Canada) at an

inspired concentration of 1.5%, titrated for a systolic blood pressure of 90–100 mmHg. In group I, a normal saline solution (0.9%) was infused at a rate of 6 mL·hr⁻¹ throughout the study period to match the amount of *iv* fluid given in group P.

The left common carotid artery was isolated and exposed over 4 cm and a 2-mm Transonic flow probe (Transonic System Inc., Ithaca, NY, USA) was placed upon the vessel and connected to a flow meter (Transonic System Inc., Ithaca, NY, USA). After a stabilization period of ten minutes (T_0), a stenosis was created using a vascular Salibi clamp placed around the common carotid artery and adjusted to produce a 10% reduction of flow (corresponding to a 75–80% stenosis). Another ten minutes was allowed for stabilization before intimal and medial injury to the artery. A standardized injury was performed by cross clamping the common carotid artery three times over ten seconds with a needle forceps holder (to the second click). All data were acquired and recorded on a Biopac data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA).

The first period of observation (Ob_1 , 20 min) began immediately after the arterial damage. Rabbits had to present at least three spontaneous CFR (sCFR) to be included in the study. Spontaneous cyclic flow reductions were defined as progressive reduction of carotid artery blood flow, followed by a sudden return to the pre-reduction level without shaking the Salibi clamp to help dislodge the thrombus (Figure 1). If less than three sCFR occurred during this period, an ipsilateral arterial lesion was repeated. If then again less than three sCFR were noted, the same protocol was applied to the contralateral carotid artery. If three or more CFR occurred during the initial observation period, the animal was included in the study and the occurrence of CFR was observed for another 70 min (Ob_2) (Figure 2).

An induced CFR (iCFR) was defined as restoration of flow velocity (≥ 5 mL·min⁻¹) after a shaking maneuver: flow velocity was assessed continuously and each time the velocity decreased below 15 mL·min⁻¹, gentle shaking of the vascular clamp was permitted to dislodge the thrombus. This well accepted maneuver may restore flow velocity and avoid complete thrombosis of the artery. A complete thrombosis was defined as a flow velocity of less than 5 mL·min⁻¹ not restored by shaking. The CFR tracings were analyzed off line by a blinded investigator unaware of the sequence of acquisition, and incidence of sCFR, iCFR and thrombosis were noted. The primary outcome of interest was the total number of CFR (both spontaneous and induced) observed during periods Ob_1 and Ob_2 .

FIGURE 2 Experimental design. Hemodynamic variables and arterial blood gases were obtained at T_0 . Hemoglobin, hematocrit, platelet count and bleeding time were obtained at T_0 and T_1 . The animal was included in the study if $\geq$ three cyclic flow reductions (CFR) were present during Ob_1 . Number of CFR and incidence of thrombosis were observed during Ob_1 and Ob_2 . St = stabilization. Ob = observation period.

Arterial blood samples were withdrawn before the first period of observation (T_0) and after completion of Ob_2 (T_1). Arterial blood gases were measured at T_0 to assess the adequacy of ventilation and oxygenation. Hemoglobin, hematocrit and platelet count were measured. A bleeding time was performed at T_0 and T_1 using the following technique: after cleaning and shaving the left ear, a standardized incision of 5 mm length and 1 mm depth was made using a Triplett bleeding time test device (Helena Laboratories, Beaumont, TX, USA). Blood drops were collected on a filter paper disc every 30 sec. Bleeding time was measured from the time to incision to the time when no blood was noted on the paper disc.

Continuous variables are presented as means $\pm$ one SD. Discrete variables are expressed as medians with ranges. Non-parametric results were analyzed with the Wilcoxon rank sum test whereas continuous results were compared to each other with a Student's *t* test. Dichotomous variables were analyzed using Chi-square test. A $P < 0.05$ was considered significant.

Results

Of the 20 rabbits initially included in the experimental protocol, eight animals were excluded (see Figure 3 for details). Five rabbits were included in group P and seven rabbits in group I. Inclusion rate was 63.6% for group I and 55.6% for group P ($P = NS$). Table I shows preoperative and intraoperative variables. The temperature was lower in the barbiturate group but within the preset value. Rabbits in group P had a 24% higher systolic blood pressure than those in group I during the first ten-minute stabilization period. The hematocrit values, platelet counts and bleeding times were similar in both groups at baseline and at the end

FIGURE 3 Flow diagram. c.a. = carotid artery; CFR = cyclic flow reduction.

TABLE I Preoperative and intraoperative variables

	<i>Pentobarbital</i> (<i>n</i> = 5)	<i>Isoflurane</i> (<i>n</i> = 7)
Weight (kg)	2.9 ± 0.2	2.9 ± 0.3
Temperature (°C)	38.1 ± 0.2	38.8 ± 0.3*
Arterial blood pressure (mmHg)	110 ± 4	89 ± 9*
Heart rate (beats·min ⁻¹)	324 ± 30	310 ± 18
Carotid blood flow (mL·min ⁻¹)	78 ± 10	69 ± 16
pH	7.49 ± 0.05	7.46 ± 0.08
PaO ₂ (mmHg)	552 ± 17	534 ± 23
PaCO ₂ (mmHg)	41 ± 6	38 ± 5

Average systolic blood pressure, heart rate and carotid blood flow during the first ten minutes period of stabilization (*T*₀). Values expressed as means ± SD. **P* < 0.05 *vs* pentobarbital.

TABLE II Hematological variables at baseline / end of study period for both groups

	<i>Baseline</i>	<i>End of study period</i>
<i>Pentobarbital (n = 5)</i>		
Hematocrit (%)	37.7 ± 1.8	36.4 ± 2.5
Platelet count (G·L ⁻¹)	407 ± 91	399 ± 73
Bleeding time (sec)	93 ± 49	73 ± 15
<i>Isoflurane (n = 7)</i>		
Hematocrit (%)	37.2 ± 2.3	35.5 ± 3.3
Platelet count (G·L ⁻¹)	465 ± 95	434 ± 87
Bleeding time (sec)	111 ± 37	105 ± 38

Values expressed as means ± SD.

of Ob₂ (Table II). The number of iCFR (median) needed to prevent thrombosis was higher in the barbiturate group, but this difference did not reach statistical significance (Table III). The total number of CFR observed and the duration of the period where CFR occurred were similar in both groups (Table III). After 30 min of observation, 75% of all animals still developed CFR. After 45 min, CFR persisted in one third of all animals (Figure 4). The incidence of

TABLE III Cyclic flow reductions (spontaneous or induced) in each rabbit

<i>Pentobarbital</i>	<i>sCFR</i> (<i>n</i>)	<i>iCFR</i>	<i>Thrombosis</i>	<i>Duration of CFR (min)</i>
1	16	0	N	61
2	4	2	Y	16
3	5	3	N	33
4	9	3	Y	47
5	13	0	N	36
Median / Mean ± SD	9	2	2	39 ± 17

<i>Isoflurane</i>	<i>sCFR</i> (<i>n</i>)	<i>iCFR</i>	<i>Thrombosis</i>	<i>Duration of CFR (min)</i>
1	14	0	N	36
2	6	1	N	45
3	5	0	N	13
4	10	3	N	30
5	9	4	N	32
6	7	0	N	18
7	14	0	N	90
Median / mean ± SD	9	0	0	38 ± 25

Number of sCFR and iCFR expressed as median; Duration of CFR expressed as mean ± SD. Y = yes; N = no; CFR = cyclic flow reduction (spontaneous or induced).

FIGURE 4 Percentage of animals in which cyclic flow reductions are still occurring over time.

thrombosis was not different in group P (2 events) compared to group I (0 event, *P* = 0.15; Table III).

Discussion

We have shown that maintenance of anesthesia with isoflurane or pentobarbital results in the same number of CFR over a similar time span. Thus, despite the use of different agents to induce and maintain anesthesia, the two models appear to produce similar results with regard to CFR. Contrary to our initial hypothesis, a platelet inhibitory activity of isoflurane-based anesthesia does not appear to be clinically relevant in this animal model.

Studies assessing the impact of isoflurane on platelet function have yielded results similar to ours. In an animal model of coronary artery stenosis in dogs, Bertha *et al.* demonstrated that halothane, but not isoflurane or enflurane, was associated with the abolition of spontaneously occurring and epinephrine induced CFR at equivalent arterial blood pressure values.¹¹ The authors speculated, based on *in vitro* studies, that halothane exerted this effect because of its increased platelet adenylate cyclase and intraplatelet cyclic adenosine monophosphate activity. In an *in vitro* study performed on blood samples obtained from patients under general anesthesia, Dogan and colleagues showed that sevoflurane and propofol, but not isoflurane, possess significant inhibitory effects on platelet aggregation.¹² Similar results were also obtained by Hirakata *et al.* as they demonstrated that in patients anesthetized with sevoflurane, but not isoflurane, secondary platelet aggregation could not be induced by adenosine diphosphate (ADP) and epinephrine.¹³ However, the same group demonstrated *in vitro* that although isoflurane was less potent than halothane and enflurane, it could inhibit thromboxane A₂ induced platelet aggregation in a dose-dependent manner. There was a 50% inhibition with a concentration of 15.7 mM (corresponding to 24 minimum alveolar anesthetic concentration, roughly 20 times the usual concentration in humans) added in the platelet preparation.¹⁴ Also, a study by Fauss *et al.* showed that there was a statistically significant inhibition of ADP-induced platelet aggregation with both nitrous oxide (N₂O) 80% and isoflurane 1.5% *in vitro* and with the combination of N₂O 60% and isoflurane 1.2% *in vivo*.¹⁵ Overall, these results tend to imply that isoflurane does not possess a clinically significant platelet inhibitory potential when used at clinically relevant concentrations.

Studies assessing the effect of barbiturates on platelet function are more confusing. The inhibitory effect, when present, is dependent upon the molecule and the animal model used. In addition, *in vitro* and *in vivo* studies have often generated divergent results. Gentry *et al.* demonstrated that a commercial preparation of pentobarbital containing 40% propylene glycol in a saline solution resulted in a marked increase in procoagulant activity in dogs, as reflected by a decrease in whole blood clotting time and in the activated partial thromboplastin time.¹⁶ However, there was no influence on the extrinsic clotting system and on platelet count and functional activity. These results were likely explained by the red blood cell lysis caused by propylene glycol. Two *in vitro* studies demonstrated enhanced secondary platelet

aggregation induced by barbiturates and mediated by modifications in the arachidonic acid pathway. Kitamura *et al.* demonstrated an increase in platelet activity with thiopental in human platelets.¹⁷ Sato and colleagues, however, demonstrated in the same *in vitro* study that thiamylal enhanced secondary platelet aggregation induced by ADP and epinephrine but that pentobarbital suppressed it.¹⁸ These results were likely explained by an increase of the arachidonic acid release by thiamylal and by a decrease of its release by pentobarbital. The discrepancies between barbiturates concerning their effects on platelet function have been evaluated more thoroughly by the same group and the authors concluded that the platelet effects were correlated with anesthetic potency, lipid solubility and chemical structure.¹⁹ Opposite results were obtained in other studies. O'Rourke *et al.* demonstrated that thiamylal, pentobarbital and barbital could inhibit *in vitro* platelet aggregation initiated by ADP and collagen in a dose-dependent manner.²⁰ This was explained by impairment of calcium-dependent platelet activation mechanisms. Dordoni *et al.* have shown that, in patients undergoing thyroid surgery, thiopental inhibits platelet function in a dose-dependent fashion while fentanyl or propofol had no effect.²¹ They also showed that thiopental had no effect on thromboxane generation, and that it reduced collagen but not ADP-induced platelet aggregation *ex vivo*. A clinical study in patients undergoing infrainguinal vascular surgery found that thiopental, although less potent than etomidate, had significant *in vitro* and *in vivo* platelet inhibitory properties.²² Similarly, Folts and Levine demonstrated *in vivo* that decreasing the amount of sodium thiamylal administered to dogs resulted in less inhibition of platelet and myocardial function.^A

We observed during the initial development of our rabbit model, that CFR decreased progressively and were abolished after 45 to 60 min of anesthesia. Yet, it has been stated that using Folts' model of arterial stenosis and intimal damage, periodic thrombosis would occur for hours providing that no antiplatelet agents were given.² Until now, few published data have addressed specifically the stability of this arterial cyclical flow model over time. Hill and colleagues, in their initial effort to develop Folts' model in rabbits, claimed to have obtained CFR during a period of time averaging 8 ± 3 hr in five anesthetized rabbits. Details on the experimental protocol and anesthetic technique are lacking since the data were never published

A Folts JD, Levine RL. Effects of barbiturate anesthesia on *in vivo* platelet thrombus formation and regional myocardial functions. Fed Proc 1984; 43: 420 (abstract).

other than in abstract form.^B In rabbits, most of the experimental protocols reported in the literature were designed to be of short duration (45 to 110 min).^{5-7,9,23} Despite their relatively short duration, most of these studies showed a trend towards a decrease in the number of CFR occurring in the control group as the experimental protocol proceeded.^{3,5-7,9}

In the present study, the average duration of CFR occurrence was approximately 40 min for both anesthesia protocols. The time span over which CFR occurred was quite variable for each rabbit, despite the same strict control of major biological variables in both groups and the standardized carotid artery stenosis and lesion. The brief stability of this rabbit arterial cyclical flow model over time could have a physiological explanation. The normally functioning hemostatic system is designed to restore vascular integrity after an endothelial injury. Primary and secondary hemostasis result in fibrin deposition at the site of the vessel injury, preventing further contact of tissue factor with the circulating platelets and coagulation factors, hence inhibiting thrombosis. The time required for fibrin deposition to be complete has not been established precisely, but could be around 45 min (Georges Étienne Rivard, hematologist, Centre Hospitalier Universitaire Mère-Enfant, Montreal, personal communication).

Obviously, this experiment has limitations. Limitations relate to the number of animals and to the absence of a complementary evaluation of platelet function. The number of animals is limited but the wide SD around the measures suggests that the model would not have been precise enough to detect experimentally relevant differences in anti-platelet activities of different anesthetic agents. Assuming an α of 0.05, a β of 0.8 and an SD of 20 min for the duration of CFR, a sample size of 64 animals would have been required to detect a between group difference of ten minutes (which is large, considering that the differences in anesthetic agents are likely to be subtle). Such a finding would not have been relevant experimentally as it would have been unlikely to contaminate the results of experiments in which an active treatment is administered and results in profound hemostatic effects. We did not examine platelet function in the present study and this may have been an oversight. In retrospect, any differences between anesthetic regimens would have been, at best, modest and might not have been picked up by the relatively simple platelet

function tests available to us, such as aggregometry or thromboelastography.

On the other hand, strengths of our study include an attempt to validate Folts' model in rabbits and the use of stringent criteria to define CFR. The experimental protocol is exactly the same as that described by Marret *et al.*⁶ As mentioned previously, our initial observations were contrary to published reports where the model is purported to be stable for prolonged periods of time.^{1,2} Since, in order to comply with the Canadian Council on Animal Care guidelines,¹⁰ we were using a different anesthetic technique, our impression was that anesthesia *per se* might be responsible for the observed discrepancy within the rabbit model. Apparently, this is not the case while discrepancies between models may relate to the animal species used (dogs, pigs, rabbits) or to the site of vessel injury (coronary, femoral or carotid artery). In our laboratory, a single operator conducted all experiments and stringent criteria were used to characterize CFR. Induced cyclic flow reductions in particular were clearly defined by measures of flow in the carotid artery. The balance between platelet accretion, subsequent gradual formation of a platelet plug in a stenosed artery and, ultimately, embolization of the platelet thrombus because of blood flow is extremely delicate. "Shaking" of the clamp is a well accepted maneuver in this model and allows the investigator to distinguish between an impending and a permanent thrombosis of the artery. Yet, to avoid any misunderstanding, we report sCFR and iCFR separately. Using this model, this is the first time CFR have been reported in this manner.

Based on the results of our study, we conclude that this rabbit model of arterial stenosis, intimal damage and CFR is not affected by the choice of anesthetic regimen and remains stable only over a short period of time. Our suggestions are twofold. First, the most effective and humane anesthetic technique should be used. Second, the ideal duration of an experimental protocol using this model should range between 30 and 45 min in order to maximize the number of animals still developing CFR towards the end of the experiment.

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