Comparative Medicine
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Vol 64, No 6
December 2014
Pages 471–477

Original Research

Noninvasive Assessment of Pulse-Wave Velocity and Flow-Mediated Vasodilation in Anesthetized Göttingen Minipigs

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Few methods for noninvasive assessment of arterial stiffness and endothelial dysfunction in porcine models are available. The aim of this study was to evaluate methods for assessment of arterial stiffness and endothelial dysfunction in anesthetized Göttingen minipigs. Pulse-wave velocity (PWV) was assessed in male Göttingen minipigs (*n* = 8; age approximately 60 wk) by using applanation tonometry of the carotid and femoral arteries. In addition, flow-mediated vasodilation (FMD) was assessed by using vascular ultrasonography of the brachial artery to evaluate endothelial dysfunction. To evaluate the reproducibility of the methods, minipigs were anesthetized by intravenous infusion of ketamine and midazolam and examined every other day for a total of 3 trials. Neither examination day nor systolic, diastolic, or mean arterial blood pressure statistically influenced PWV or FMD. The median interexamination coefficient of variation was 17% for PWV and 59% for FMD. Measured values of PWV corresponded largely to those in clinically healthy humans, but FMD values were lower than expected for lean, young animals. Although the ketamine–midazolam anesthesia we used has been associated with minor hemodynamic effects in vivo, in vitro studies suggest that both drugs are vasodilatory. Therefore anesthesia might have influenced the endothelial response, contributing to the modest FMD response and the concurrent high coefficients of variation that we noted. We conclude that PWV—but not FMD—showed acceptable interexamination variation for its potential application in porcine models.

Abbreviations: FMD, flow-mediated vasodilation; FVI, integrated flow velocity; GTN, glyceryl trinitrate; PWV, pulse-wave velocity; T, transit time.

Cardiovascular disease has become a global challenge in public health,[42] and the development and characterization of comparative animal models are of increasing importance. Several animal models of atherosclerosis, including porcine, have been described.[11,12,34] Due to similarities to humans in the anatomy of the cardiovascular system and metabolic physiology, pigs represent a generally useful model in regard to preclinical evaluation and pharmacology.[36] Assessment of changes related to atherosclerosis in vivo would be valuable in for example longitudinal assessment of drug effect, but few noninvasive methods for evaluating structural and functional changes in the arteries of pigs are available. In humans, increased arterial stiffness, which occurs with advanced age, also is caused by the pathophysiologic changes associated with atherosclerosis,[26] and noninvasive methods for assessing arterial stiffness have been established. The evaluation of pulse-wave velocity (PWV) by using pressure transducers, such as applanation tonometry, is a method recognized as an independent predictor for cardiovascular events in epidemiologic studies.[21] The method evaluates the velocity with which the pulse wave is propagated through the arterial tree, with arterial stiffness causing increased velocity.[1,22,29,37,39] Flow-mediated vasodilation (FMD), assessed by vascular ultrasonography, represents a noninvasive evaluation of endothelial-dependent vasodilation. A decrease in vasodilation as a response to increased shear stress has been recognized as a marker of endothelial dysfunction, which precedes the development of atherosclerosis.[5,7] Recent studies have shown that the FMD method is applicable in large animals (that is, dogs and horses) and that a decreased FMD response occurs in dogs with valvular heart disease.[10,15,23,28]

The aim of this study was to evaluate the reproducibility of methods for assessing arterial stiffness (PWV) and endothelial function (FMD response) in anesthetized Göttingen minipigs, including the influences of arterial blood pressure, heart rate, and room and body temperatures on these methods.

Materials and Methods

The studies involved 8 male, intact Göttingen minipigs (*Sus scrofa*) bred at Ellegaard Göttingen Minipigs A/S (Dalmose, Denmark; www.minipigs.com); pigs had a median age of 59 wk (interquartile range, 58 to 60 wk) and median body weight of 28.6 kg (interquartile range, 27.6 to 29.9 kg). Minipigs were housed

Received: 21 Feb 2014. Revision requested: 02 Apr 2014. Accepted: 08 May 2014.
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individually, with a 12:12-h light:dark diurnal cycle, a relative humidity of 50% to 70%, and a room temperature of 22 to 24 °C. They were fed a standard minipig chow (Minipig Diet, Special Diet Services, Essex, UK) and had free access to water and bedding material. Permanent double-lumen intravenous catheters (Redo TPN Catheters, William Cook Europe ApS, Bjæverskov, Denmark) were implanted as previously described.[20] The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

Anesthesia protocol. Anesthesia was induced by using midazolam (0.33 mg/kg IV; Hameln Pharmaceuticals, Hameln, Germany) followed by ketamine (13 mg/kg IV; Intervet, Boxmeer, the Netherlands). Minipigs were maintained under anesthesia by constant intravenous infusion of midazolam (1.5 mg/kg/h) and ketamine (33 mg/kg/h).[35] During anesthesia, minipigs breathed room air spontaneously and were not intubated. The colors of skin and mucosal membranes were monitored subjectively throughout the procedure. Minipigs were monitored by ECG by using a triangular lead system,[25] with integrated ECG units in the ultrasonograph and the tonometry device (both described following). Blood pressure and pulse rate were measured by using an oscillometric blood pressure device (Cardell 9301V, CAS Medical Systems, Branford, CT) with a sphygmomanometric cuff applied to the left antebrachium, according to veterinary guidelines.[3] Before tonometry of the carotid artery and ultrasonography of the brachial artery for FMD, systolic, diastolic, and mean arterial blood pressures and pulse rate were recorded. Rectal and room temperatures were measured at the beginning and end of each examination.

Experimental set-up. Minipigs were fasted a minimum of 8 h before the procedure, and evaluations were performed in a temperature-controlled room as recommended.[7,21] Each animal was examined once every other day for a total of 3 evaluations. Four minipigs were examined each day, in the same order and starting at approximately the same time. Animals were examined beginning at 15 to 20 min after anesthesia induction, at which time stable hemodynamics were observed under constant IV infusion of ketamine and midazolam. Minipigs first were positioned dorsally for tonometry of the carotid artery and then laterally for tonometry of the femoral artery and measurements of endothelial-dependent and -independent vasodilation by ultrasonography of the brachial artery at the right brachium. Tonometry and assessment of FMD was interspersed with data collection of 10 to 20 min in duration for an unrelated study (data not shown). Overall, each examination lasted a median of 90 min (interquartile range, 85 to 97 min). Measurement of PWV was limited to 5 min per examination site per observer, and assessment of FMD, including nitroglycerin application, lasted approximately 25 min.

Vascular ultrasonography was performed by a single observer (TPL) with previous experience of more than 100 vascular veterinary ultrasonography recordings in anesthetized pigs. PWV measurements were performed by 2 observers (ATC and CJJ) with hands-on experience from pilot experiments in 2 anesthetized pigs.

PWV. The pressure pulses of a central artery (the right common carotid artery) and a peripheral artery (the right femoral artery) were palpated cranial to the manubrium sterni and at the medial condyle of the right femur, respectively. By using an applanation tonometer with an integrated ECG unit (PulsePen, DiaTecne, Milan, Italy), arterial pressure pulse waves and a simultaneous ECG recording were obtained from each anatomic location, capturing a maximum of 6 valid pressure pulses within a maximum of 5 min. After the acquisition of all recordings from all animals, transit times (T; the delay between the R-segment of the ECG and the foot of pulse wave) were calculated automatically by using tonometry software. All pulse waves in the recordings were controlled manually for adequate quality and correct detection of the foot of each wave. Pulse waves of inadequate quality and recordings with erroneous detection of foot of wave were discarded. For the central and peripheral locations (T_1 and T_2, respectively), an average T was calculated from valid recordings (maximum, 6). The indirect distance between examination sites was measured by using a caliper from the proximal part of the manubrium sterni to the caudal region of the great trochanter of the right femur. For calculation of the PWV (Figure 1), the distance was divided by the difference between the femoral and carotid transit time [$PWV = L/(T_2 - T_1)$].[30] Recordings from a single observer (ATC) were used for evaluating interexamination variation, and recordings from 2 observers (ATC and CJJ) on examination day 2 were used to evaluate interobserver variation.

FMD. For assessment of FMD, minipigs were placed in a right lateral position, as previously described.[24] A sphygmomanometric cuff was placed at the right midantebrachium, and a 13-MHz linear-array transducer of (model 12L-RS, GE Medical Systems, Brøndby, Denmark) was applied to the brachial region. The transducer was manually held in place throughout baseline and postintervention recordings. The ultrasonography system (Vivid I, GE Healthcare) was used to obtain a longitudinal 2D cine loop of the middle part of the right brachial artery, with a visually clear image of the near and far wall of the artery. For assessment of blood flow velocity, a pulsed-wave Doppler recording signal was recorded (maximal angle of insonation, 60°). A 30-s 2D recording of the baseline diameter and a 15-s pulsed-wave Doppler recording of the baseline blood flow velocity were obtained.

After baseline recordings were completed, ischemia was induced by inflation of the sphygmomanometric cuff for 4.5 min to a minimum of 100 mm Hg above systolic blood pressure. To ensure sufficient ischemia during inflation, reduced blood flow was monitored subjectively during cuff inflation by using pulsed-wave Doppler. Immediately after decompression, the pulsed-wave Doppler signal was recorded for 10 s for postcompression blood flow assessment, followed by 2D recording of the diameter for 120 s. A second baseline recording was obtained after a 10-min 'washout' period,[7] followed by sublingual application of 0.4 mg glyceryl trinitrate (GTN; Nitrolingual, Pohl Boskamp, Hohenlockstedt, Germany). After 3 min, a 120-s recording of the diameter of vessel was obtained to evaluate endothelial-independent vasodilation. To ensure assessment of approximately the same arterial segment during each examination of each subject, anatomic landmarks in the region were noted.

Offline analyses were performed by using a semiautomated border-detection program (Brachial Analyzer for Research, Medical Imaging Applications, Coralville, IA). In each frame, arterial segments, including a visually well-defined near and far wall of the artery, were defined manually as the area of interest and was followed by automated border detection throughout the entire recording. The tracking quality of the automated border detection was controlled manually for each frame, and incorrectly detected borders of the vessel wall were discarded. For all examination days, all accepted frames (43% to 66% of the total number of

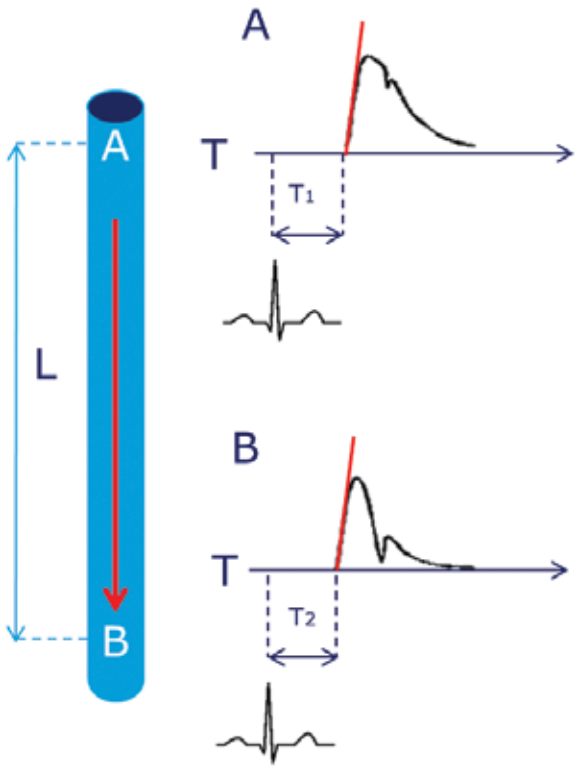

Figure 1. Calculation of pulse-wave velocity (PWV) from pressure waves and transit times (T) from a central and peripheral artery. Pressure waves and concurrent QRS complexes from the central location, the carotid artery (A), and the peripheral location, the femoral artery (B). The red arrow indicates direction of flow from the central to the peripheral artery. PWV = $L/(T_2 - T_1)$. L, distance between measuring sites; T_1, transit time in the carotid artery; T_2, transit time in the femoral artery. Modified from http://www.diatecne.com/

frames during baseline and post compression recordings) were included in the analysis of the FMD and GTN responses. Baseline diameter was calculated as the average diameter calculated from the accepted frames from the baseline recording. The maximal luminal diameter after decompression (FMD response) or GTN application (GTN response) was defined as the mean of the peak 10 frames (peak frame + 5 adjacent frames on each side of peak frame) in the recordings. FMD and GTN responses were defined as: [(maximal luminal diameter – mean baseline diameter) / mean baseline diameter] × 100%.

The change in blood flow velocity from baseline to after compression was assessed as the flow velocity integral (FVI) through analysis of the pulsed-wave Doppler signals by using EchoPAC software (version 7.0.0, PC Dimension, GE Medical Systems). An average of 5 consecutive pulsed-wave Doppler signal waveforms before ($FVI_{Baseline}$) and after ($FVI_{Postcompression}$) vessel compression were used to calculate the percentage difference in blood flow: FVI (%) = ($FVI_{Postcompression} - FVI_{Baseline}$) / $FVI_{Baseline}$ × 100%.

Statistics. Results are presented as medians and interquartile intervals (25% and 75%) unless otherwise stated. To describe the repeatability of the methods, the interexamination coefficient of variation (defined as CV [%] = 1 SD / mean × 100%) was calculated for PWV, baseline arterial diameter, FMD response, GTN response, and FVI. Furthermore, interobserver variation was calculated for PWV from the second examination day. For methods with acceptable levels of interday variation (CV < 20%), Bland–Altman plots of interobserver and interexamination were added, to evaluate agreement between examinations. The influence of MAP, SAP, DAP, pulse rate, examination day, room and body temperature on PWV, FMD response, and GTN response were tested by using a linear mixed model, with animal number as a random effect. Average values of 2 measurements of blood pressures (systolic, diastolic, and mean arterial), heart rate, and body and room temperatures (before and after the examination) were used in the statistical analysis. Residuals were tested for normality and homogeneity of variation. A P value of less than 0.05 was considered significant.

All statistical analyses were performed by using JMP (version 8.0.1, SAS Institute, Cary, NC), and graphs were generated by using Prism software (version 5.03, Graph Pad Software, La Jolla, CA).

Results

Neither arterial blood pressure (mean, systolic, diastolic), pulse rate, examination day, room, nor body temperature significantly influenced PWV, FMD response, or GTN response in the male Göttingen minipigs that we tested (Table 1). Figure 2 illustrates an example of the output from the tonometry software.

Values for PWV were obtained from 5 of the 6 minipigs examined; the remaining pig was excluded from statistical analysis due to missing values on examination day 2. The median interobserver and interexamination coefficients of variation were 17% (range, 8% to 23%) and 17% (range, 3% to 23%), respectively. Bland–Altman plots of interexamination variation and interobserver variation for PWV show that the interexamination difference (Figure 3 A) is distributed close to 0 and illustrate an acceptable agreement between examination days 2 and 3. Interobserver difference (Figure 3 B) indicates a trend (P = 0.11) toward higher values from observer 2 than observer 1, although the difference is not significantly different from 0.

Regarding FMD and GTN responses in the brachial artery, recordings of baseline diameter before vessel compression yielded low median interexamination variation (4% (2-6%)). However, the median interexamination coefficient of variation for the FMD response was 59% (39-184%), whereas that for the GTN response exceeded 100% (median value of 204% (85-306%). Blood flow recordings using pulsed-wave Doppler signals were acquired from all minipigs on all examination days with an interexamination variation of 34% (14-60%). However, due to technically related artifacts in the recordings, the software could not estimate FVI in one or more examinations from 4 animals, which were therefore excluded from further analysis.

Discussion

In this study, we evaluated PWV and FMD as methods to assess arterial stiffness and endothelial dysfunction in anesthetized Göttingen minipigs. Although both methods proved useful, only PWV demonstrated, even with little practice, acceptable levels of interexamination and interobserver variation. The same observers repeatedly examined at the same time of day a homogenous group of animals in regard to weight and age, and the methods were modified according to recommended standards.[7,21] Values of PWV corresponded largely to what has been reported for clinically healthy young humans, with values below 10 m/s.[19,27]

Table 1. Cardiovascular parameters of minipigs (n = 8, unless otherwise indicated) during each of 3 examinations

	1	2	3
Arterial blood pressure (mm Hg)			
Systolic	119 (111–135)	116 (98–122)	119 (98–135)
Diastolic	63 (56–69)	58 (47–68)	68 (42–73)
Mean	89 (82–98)	84 (68–91)	91 (64–103)
Pulse rate (bpm)	76 (69–79)	70 (65–75)	74 (67–81)
Body temperature (°C)	36.9 (36.7–37.6)	37.1 (36.7–37.4)	36.8 (36.6–37.1)
Room temperature (°C)	22.9 (22.2–23.4)	23.1 (22.4–23.7)	23.5 (23.4–23.8)
PWV[a] (m/s)	6.2 (3.6–7.5)	5.9 (5.2–6.2)	6.5 (5.2–6.9)
Baseline diameter of artery (mm)	2.6 (2.5–2.6)	2.7 (2.6–2.7)	2.5 (2.5–2.6)
FMD response (%)	4.6 (–1.5–7.2)	3.0 (1.9–4.2)	5.0 (2.9–9.6)
GTN response (%)	5.7 (–0.6–10.2)	1.8 (–2.3–3.2)	0.6 (–2.9–2.8)
FVI[b] (%)	196 (106–275)	234 (122–311)	115 (66–302)

Data are shown as median (interquartile range, 25% to 75%).
[a] n = 7
[b] n = 4

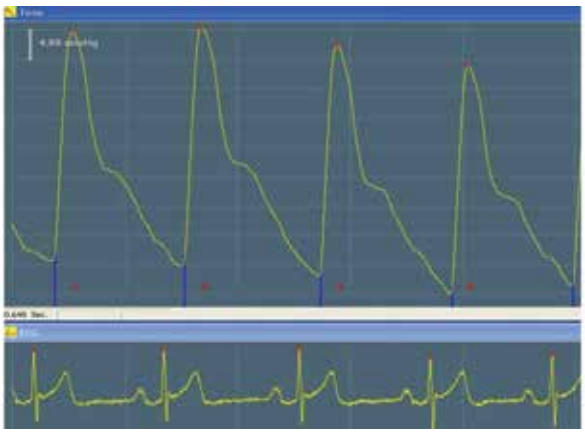

Figure 2. Example of the output from the tonometry software. The red dots mark the R segment of the ECG and the top of the pulse wave. Each blue line with a number marks the foot of a wave. The tonometry software calculates the time delay (T) between the R segment and the foot of the wave as the time difference between the peak of the R segment and the blue line.

However, blood pressure is a known confounder in relation to PWV,[22,37,38] and in the current study all measures were obtained noninvasively, including blood pressure. Published data on blood pressure measurements in Göttingen minipigs are limited to invasive measures from the femoral or carotid artery, in either anesthetized or awake animals.[2,33] The blood pressure values we observed in the current study were considerably lower than what has been reported from conscious minipigs by using telemetry[33] and anesthetized pigs,[2] suggesting that anesthesia tends to decrease blood pressure. However, ketamine has a known hypertensive potential, but midazolam is expected to attenuate this effect, resulting in normotension.[40] Furthermore, in the current study, blood pressure was evaluated from the antebrachium of the minipigs, as recommended in other species.[3] The antebrachium of Göttingen minipigs is somewhat conical in shape, which compromises uniform compression of the vessel and potentially causes underestimation of blood pressure. This underestimation is a more plausible explanation of the low values observed.

The potential hemodynamic effect of the anesthetics applied in our study also presents an important consideration in regard to FMD. Many factors are known to influence the variability of the FMD response in human patients, including room temperature and sex,[9,13] but patients are always examined unanesthetized. Despite the low baseline variation (CV = 4%) in our data, a predilated vessel would explain the reduced or absent endothelial-dependent and -independent response that we noted, given that both values are calculated as ratios and therefore sensitive even to slight changes in hemodynamics. Although the anesthetic combination we used was intentionally selected to avoid marked hemodynamic alterations,[14,23] in vitro studies suggest a vasodilatory effect of both ketamine and midazolam in porcine and rodent arterial segments.[4,6,16,17] Another potential indirect effect of anesthesia is related to respiration. Considering that the minipigs in this study were observed only subjectively in relation to respiration and were not supplied with oxygen, a potential hemodynamic effect as a consequence of hypoxia cannot be excluded. However, the initial effects of hypoxia would be hypertension and an increase in heart rate[31]—neither of which observed in any of our animals at any point during the study. Furthermore, ketamine has little or no depressant effect on respiration or the respiratory response to hypercapnia when the drug is supplied slowly intravenously.[40] In addition, low room temperature is thought to lead to decreased microcirculation and therefore a reduced FMD response.[41] In the current study, room temperature ranged between 22.9 and 23.8 °C (Table 1), which is within the range (20 to 25 °C) at which no effect on microcirculation is expected.[32]

The use of a stereotactic probe-holding device is recommended in human studies.[7] In the current study, we used a handheld probe in light of pilot data indicating that the artery can migrate from the original measuring site during recording. However, this use of a handheld transducer and the possibility for dislocation of the artery during recording may have contributed to the variation we observed in the FMD and GTN responses.[8] Although it is not recommended to compare FMD values across laboratories, the data obtained in the current study (range, 3% to 5%) are low compared with measurements from clinically healthy humans (range, 5% to 15%).[9,32] Furthermore, image acquisition of small artery diameters (diameter, <2.5 mm) is known to be technically challenging,

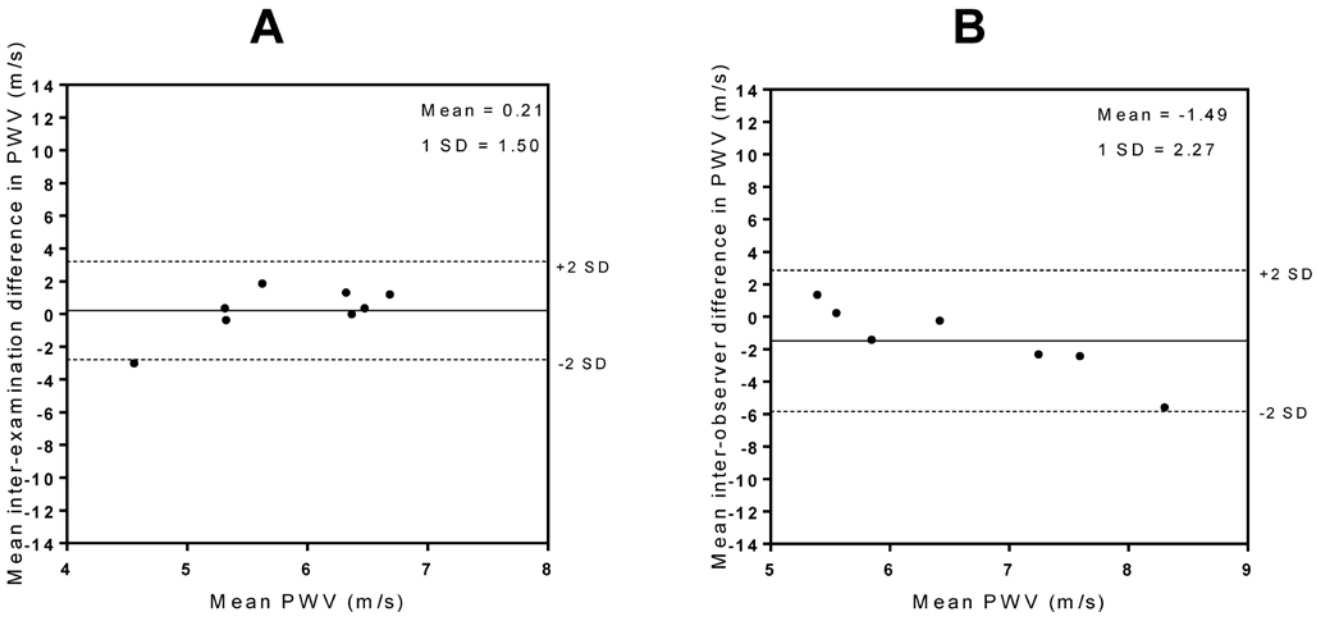

Figure 3. Bland–Altman plots of (A) the interexamination difference between examination days 2 and 3 and (B) the interobserver difference between 2 observers on day 2 of PWV measurements.

and the expected greater response due to the smaller artery size[7] was not realized. In comparison, the brachial artery diameter in both female and male adult humans is more than 3.0 mm.[8] Interestingly, a recent study on evaluation of FMD in the rearlimb of nonhuman primates (*Macaca fascicularis*) reported FMD responses ranging from 6% to 30%.[18] In addition, the cited study[7] used the same anesthetic combination (albeit given intramuscularly) as given in the present study and was able to obtain plausible vasodilatory responses. We avoided the use of intramuscular dosing of ketamine and midazolam in light of the risk of local tissue damage due to the low pH of the formulations. The difference in the route of administration likely does not explain the observed differences in FMD between nonhuman primates and minipigs. More importantly, because of the PWV recordings, the minipigs in the current study were anesthetized for a longer period of time before FMD measures than were the macaques in the previous study.[7] We chose the order for the experimental set-up to avoid a potential effect of GTN application on PWV measures.

In regard to future assessments of PWV in pigs, some aspects can be improved. Including invasive measures of blood pressure would be an important contribution to the assessment. Furthermore, the measurement of pressure pulses in the central and peripheral arteries could be obtained immediately one after the other or simultaneously by applying 2 pressure transducers at the same time. Assessment of each pulse wave immediately after acquisition rather than at the end of the experiment is another approach to ensuring the capture of sufficiently high-quality pulse waves.[22,37,38] Moreover, the measurement of pressure pulses from regions containing excess subcutaneous fat is a known challenge in human patients.[38] Obese minipigs tend to store excess fat in the cervical region, thus complicating the measurement of pressure pulses from the carotid artery. This drawback may limit the usefulness of the method in very obese animals. In future studies of FMD, several aspects to consider include both direct and indirect potential pharmacologic effects on vessel tone, improved surveillance of respiratory parameters, and practical options for probe-holding devices.

Limitations of the current study include the use of noninvasive blood pressure measurement methods that have not been validated against invasive techniques in minipigs. Furthermore, the need for anesthesia for this kind of study in pigs poses an inevitable hemodynamic challenge and is an important consideration when evaluating arterial function. Oxygen saturation was not assessed in the current study, and oxygen not supplied. Both of these factors could be considered in future studies. In addition, because the data regarding blood flow by FVI in 4 of 8 minipigs had to be excluded, the resulting insufficient amount of data caused the evaluation of the repeatability of this measure to be inconclusive. The FVI values from the remaining minipigs did, however, correspond to what has been reported in clinically healthy dogs.[15]

In conclusion, we determined that noninvasive evaluation of arterial stiffness using PWV measurements was feasible in anesthetized Göttingen minipigs, yielding acceptable levels of interexamination and interobserver variation. The PWV method has the potential to be applied in porcine models of atherosclerosis. FMD assessment showed large interexamination variation, most likely explained by direct and indirect hemodynamic effects of the anesthesia. Neither blood pressure, pulse rate, examination day, nor room or body temperature significantly affected either PWV or FMD data.

Acknowledgements

Novo Nordisk A/S is acknowledged for funding the animal experiments and the LifePharm Centre (In vivo Pharmacology, University of Copenhagen) for providing the PhD grant for TPL.

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