Complex porcine model of atherosclerosis: Induction of early coronary lesions after long-term hyperlipidemia without sustained hyperglycemia

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BACKGROUND: The incidence of coronary artery disease (CAD) is still increasing in industrialized countries and it is even higher in diabetic patients. For experimental studies investigating the pathophysiology of CAD, the use of an animal model comparable with the pathological situation in patients is crucial.

OBJECTIVE: To develop a model of advanced coronary atherosclerosis with induction of hyperlipidemia and hyperglycemia in domestic pigs.

METHODS: Six pigs were fed a standard pig chow (controls), two were fed a 2% cholesterol and 17% coconut fat diet (Chol group), and two pigs received a 4% cholesterol and 17% coconut fat diet combined with streptozotocin (STZ) injections to induce diabetes (High Chol+STZ group). Serum lipid and plasma glucose values were analyzed, and histochemical staining for morphometric analysis and immunohistochemistry were performed.

RESULTS: Pigs on the hyperlipidemic diet had elevated mean ($\pm$ SD) serum lipid levels (total cholesterol 5.05 ± 1.45 mmol/L [Chol] and 5.03 ± 2.41 mmol/L [High Chol+STZ] versus 2.09 ± 0.23 mmol/L [controls]). Histopathological evaluation revealed an initial stage of coronary atherosclerosis. None of the STZ-treated pigs showed a sustained elevation of plasma glucose (mean glucose before STZ injection was 5.11 ± 0.94 mmol/L and thereafter was 6.03 ± 2.39 mmol/L) or a decline in pancreatic beta cells.

CONCLUSIONS: The current data suggest that the domestic porcine model is not suitable to create severe CAD using an atherogenic diet in combination with STZ injections for experimental interventional vascular research. This may be due to different STZ sensitivities among species. However, hyperlipidemia induced early pathological lesions in coronary arteries resembling initial stages of atherosclerosis without severe luminal narrowing.

Key Words: Atherosclerosis; Coronary disease; Diabetes mellitus; Hyperlipoproteinemia

Un modèle porcin complexe d'athérosclérose : L'induction de lésions coronaires précoces après une hyperlipidémie prolongée sans hyperglycémie soutenue

HISTORIQUE : L'incidence de coronaropathie augmente toujours dans les pays industrialisés et est encore plus élevée chez les patients diabétiques. Pour mener des études expérimentales sur la physiopathologie de la coronaropathie, il est essentiel d'utiliser un modèle animal comparable à la situation pathologique des patients.

OBJECTIF : Mettre au point un modèle d'athérosclérose coronaire avancée avec induction d'hyperlipidémie et d'hyperglycémie chez des porcs domestiques.

MÉTHODOLOGIE : Six porcs ont été alimentés avec un mélange standard pour les porcs (sujets témoins), deux ont reçu un régime à 2 % de cholestérol et à 17 % de gras de noix de coco (groupe chol), et deux, un régime à 4 % de cholestérol et à 17 % de gras de noix de coco associé à des injections de streptozotocine (STZ) pour induire le diabète (groupe chol élevé+STZ). Les auteurs ont analysé les valeurs de lipide sérique et de glucose plasmatique et ont procédé à une coloration histochimique en vue d'une analyse morphométrique et d'une immunocytochimie.

RÉSULTATS : Les porcs suivant le régime hyperlipidémique avaient un taux lipidique sérique moyen élevé ($\pm$ ÉT) (cholestérol total $5,05 \pm 1,45$ mmol/L [chol] et $5,03 \pm 2,41$ mmol/L [chol élevé+STZ] par rapport à $2,09 \pm 0,23$ mmol/L [sujets témoins]). L'évaluation histopathologique a révélé une phase initiale d'athérosclérose coronaire. Aucun des porcs traités par STZ n'a démontré une élévation soutenue du glucose plasmatique (le glucose moyen avant l'injection de STZ était de $5,11 \pm 0,94$ mmol/L et, par la suite, de $6,03 \pm 2,39$ mmol/L) ou une diminution des cellules bêta pancréatiques.

CONCLUSIONS : D'après les données actuelles, le modèle de porc domestique ne convient pas pour créer une grave coronaropathie au moyen d'un régime athérogène en association avec des injections de STZ dans le cadre de la recherche vasculaire interventionniste expérimentale. Ce peut être à cause de différentes sensibilités au STZ selon les espèces. Cependant, l'hyperlipidémie induisait des lésions pathologiques précoces dans les artères coronaires, semblables aux phases initiales de l'athérosclérose, sans rétrécissement important de la lumière.

The cardiovascular system exhibits many similarities between pigs and humans. The coronary arteries in pigs and humans are muscular, which is distinctly different from the elastic rabbit iliac artery (1). In addition, pigs are able to develop spontaneous atherosclerotic

lesions with advancing age. Moreover, atherosclerosis can be induced by dietary manipulation and lesion development can be initiated by vascular injury (usually by angioplasty) (2,3). The anatomical proportions are comparable with humans, in contrast to minipigs. The

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Figure 1 Blood lipid values. Triglycerides, total cholesterol (Tot chol), high-density lipoprotein cholesterol (HDL chol) and low-density lipoprotein cholesterol (LDL chol) levels (means and SDs) in control pigs; pigs fed a 2% cholesterol and 17% coconut fat diet for 8 and 13 weeks (Chol group); and pigs fed a 4% cholesterol and 17% coconut fat diet for 13 weeks in combination with streptozotocin (High Chol+STZ group). Values were obtained by averaging the biweekly determined lipid levels in each group. Tot chol, HDL chol and LDL chol levels in the Chol and High Chol+STZ groups are noticeably greater than in the control group

porcine epicardial coronary artery distribution resembles that of humans, albeit with fewer collateral vessels (4). However, spontaneous atherosclerotic lesions are rarely seen in juvenile pigs, which does not compare with diseased human coronary arteries. Taken together, there is a lack of adequate models for atherosclerosis and it seemed conceivable that porcine coronary arteries undergo the same atherosclerotic changes as those of humans under the condition of hyperlipoproteinemia and diabetes. Therefore, the aim of our study was to develop such an animal model for appropriate future experimental research in the field of coronary artery disease (CAD) and coronary restenosis.

To create such an atherosclerotic animal model, pigs in the present study were fed a special atherogenic chow to induce hypercholesterolemia, because a number of studies have shown that hypercholesterolemia is associated with the development of atherosclerosis (5-8). Diabetes is another major risk factor for the development of atherosclerosis and, because streptozotocin (STZ) was successfully used to induce diabetes in other studies (6,9-13), we used a previously described STZ injection protocol (6).

METHODS

Animals and treatment

All studies were performed with approval from the Animal Care Committee of the Berlin State Office in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication number 85-23, revised 1996) (14). Ten male, castrated juvenile domestic cross-bred pigs (12 to 14 weeks of age with a mean [± SD] body weight of 30.7±1.9 kg) were included in the study. Six pigs were fed a standard pig chow for 13 weeks (control group). Two animals (pig 1 and pig 2) were fed a 2% cholesterol and 17% coconut fat diet (Chol group) for eight or 13 weeks, respectively. The two remaining pigs (pig 3 and pig 4) received a 4% cholesterol and 17% coconut fat diet for 13 weeks in combination with a total intravenous dose of 150 mg/kg STZ (High Chol+STZ group), split into three doses on three consecutive days to induce diabetes. For intravenous STZ injections, pigs were anesthetized with ketamine (25 mg/kg), xylazine (20 mg/kg), azaperone (4.8 mg/kg) and atropine (0.2 mg/kg) intramuscularly. Using a quick test (Akku-Check; Johnson & Johnson, Germany), plasma glucose was measured before and 6 h after each STZ injection, then once daily

from day 4 to day 7. Thereafter, glucose levels were measured once per week (up to week 8) and then biweekly.

Under general anesthesia, blood samples for lipid measurements were drawn from all pigs biweekly. Animals were sacrificed after an observation time of 13 or eight weeks, using T61 (Intervet, Germany) intravenously.

Histopathological processing

Porcine hearts were harvested immediately after sacrifice and perfused with Ringer's lactate at 100 mmHg for 10 min followed by a perfusion fixation with 10% neutral buffered formalin at 100 mmHg for 60 min via pressure tubing seated in the ascending aorta. The hearts were immersion-fixed in 10% neutral buffered formalin overnight. To preserve the adventitia and the surrounding periaortic tissues, the coronary arteries were carefully dissected and removed en bloc with the adjacent tissues, embedded in paraffin and cut at 5 µm intervals. For each representative arterial segment, at least eight serial sections were stained with hematoxylin and eosin.

Several pieces of pancreas from both of the High Chol+STZ animals and from one pig in the Chol group, were dissected and immersion-fixed in 10% neutral buffered formalin overnight. Sections were embedded in paraffin and cut into 5 µm sections.

Immunohistochemistry

Immunohistochemical labelling of macrophages was carried out using previously described methods (15). In brief, 5 µm tissue sections were dewaxed and rehydrated, and macrophages were detected by applying a 1:25 mouse antihuman macrophages antibody (Serotec, Germany).

To examine the effect of STZ injection on insulin-producing beta cells, sections of pancreas were incubated with a polyclonal guinea pig 1:100 antihuman insulin antibody (DAKO, Cytomation GmbH, Germany).

An EnVision kit (DAKO) was applied for 30 min, and alkaline phosphatase activity was visualized using a vector blue substrate kit (Vector Laboratories Inc, USA). Nuclear fast red stain (DAKO) served as a nuclear counterstain.

Lipid measurement

Lipid measurement in blood serum was carried out by the cardiological laboratory (Charité – Campus Benjamin Franklin in Berlin, Germany). Triglycerides were measured using an enzymatic test (GPO-PAP, Roche, Germany). Total cholesterol was measured with a kit (Enzymkit Chol #2016630, Roche). Cholesterol was fractionated in the centrifuge (40,000 rpm, 100,000 g) in low-density lipoprotein (LDL), very LDL, and high-density lipoprotein (HDL) cholesterol. HDL cholesterol was directly measured; LDL cholesterol was classified via precipitation.

RESULTS

Blood lipid values and body weight

As shown in Figure 1, pigs in the Chol group had higher cholesterol and triglyceride levels than the control group (total cholesterol 5.05±1.45 mmol/L versus 2.09±0.23 mmol/L; HDL cholesterol 1.82±0.76 mmol/L versus 0.73±0.23 mmol/L; LDL cholesterol 3.35±1.12 mmol/L versus 1.38±0.76 mmol/L; triglycerides 0.55±0.4 mmol/L versus 0.27±0.08 mmol/L).

As also shown in Figure 1, cholesterol levels in the High Chol+STZ group were much higher than in the control group (total cholesterol 5.03±2.41 mmol/L versus 2.09±0.23 mmol/L; HDL cholesterol 1.82±0.81 mmol/L versus 0.73±0.23 mmol/L; LDL cholesterol 3.05±1.64 mmol/L versus 1.38±0.76 mmol/L). There was no difference in triglyceride levels between the animals treated with High Chol+STZ (0.26±0.19 mmol/L) and the control animals (0.27±0.08 mmol/L).

Animals in the control, the Chol and the High Chol+STZ groups had a mean body weight of 30.7±1.9 kg at the start of the different pig chow regimens. The mean body weight of the Chol and High Chol+STZ groups increased to 84±2.4 kg after eight and 13 weeks of

Figure 2) Plasma glucose concentration in pigs fed a 4% cholesterol and 17% coconut fat diet for 13 weeks in combination with streptozotocin (STZ) (High Chol+STZ group). Plasma glucose levels of pig 3 and pig 4 in the High Chol+STZ group before and after STZ injections are shown. For the first three days, plasma glucose values were obtained immediately before (A) and 6 h after (B) each STZ injection (d1A to d3B). For the following four days, plasma glucose was measured daily (d4 to d7), and thereafter once per week (w2 to w8) and biweekly (w8 to w13). In pig 3, almost no changes in fasting plasma glucose levels were observed, whereas in pig 4, an initial increase was observed after the final STZ injection, lasting for up to seven weeks, which returned to baseline values by week 13. Black arrows indicate STZ injections. d Day; w Week

the atherogenic diet and STZ injections compared with a mean body weight of 76 ± 1.8 kg in the control group.

Plasma glucose levels in the High Chol+STZ group

Basic fasting plasma glucose levels of the animals in the High Chol+STZ group are summarized in Figure 2. The mean fasting plasma glucose levels after the initiation of STZ injections were 4.98 ± 0.94 mmol/L (pig 3) and 7.07 ± 2.92 mmol/L (pig 4). In pig 3, almost no change in plasma glucose levels occurred after the second STZ injection (except for a single drop below 2.5 mmol/L). In pig 4, an increase in plasma glucose was observed after the final STZ injection, with values between 6.6 mmol/L and 12.5 mmol/L, lasting for up to seven weeks, which returned to baseline by week 12. In summary, no continuous increase in plasma glucose was achieved after STZ injections in the present experiment.

Histopathology

In contrast to coronary arteries of the control group, which had no atherosclerotic changes of the arterial wall (data not shown), the histopathological evaluation of the harvested and prepared vessel samples from the Chol and High Chol+STZ groups revealed fatty streaks (Figure 3) that contained foam cells, probably derived from recruited macrophages (Figure 4). In both diet-fed groups, infiltration of macrophages was limited to the inner layers of the coronary arteries (tunica intima and media), whereas no macrophage infiltration was found in any of the arterial compartments in the control group (data not shown). No plaques were noticeable.

The number of pancreatic beta cells in all three examined pigs did not vary noticeably (Figure 5). In fact, pigs treated with STZ (High Chol+STZ) did not have fewer beta cells than the non-STZ-treated pig.

DISCUSSION

The present experiment was designed to establish a model of advanced coronary atherosclerosis in domestic pigs through induction of hyperlipidemia and hyperglycemia. Although a high-cholesterol and high-fat diet caused elevated cholesterol serum levels throughout the observational period, early but not advanced coronary lesions were induced.

Figure 3) Early lesion. **A** Representative cross-section of the right coronary artery of pig 3 after being fed a 4% cholesterol and 17% coconut fat diet for 13 weeks in combination with streptozotocin therapy (hematoxylin and eosin staining, original magnification $\times 40$). **B** Cutout of **A**. Black arrows indicate foam cells and fatty streaks (hematoxylin and eosin staining, original magnification $\times 400$)

STZ injections failed to markedly increase blood glucose and neither histomorphological characteristics of the coronary lesions nor the lipoprotein profile were altered. The results of the present study suggest that an atherogenic diet may lead to initial stages of CAD but it also indicates that it is not possible to create a reproducible diabetic and severely atherosclerotic domestic pig model suitable for interventional research in cardiology or vascular medicine.

The growing incidence and morbidity of patients with CAD, often complicated by one of its major risk factors (diabetes), has indicated an increasing need to develop a better animal model of accelerated atherosclerosis possibly associated with diabetes. Since the early 1980s, several groups have worked on this issue, initially with diet-induced atherogenesis in small- and large-animal models, followed by diabetes models in rodents, up to recent experiments in the porcine model in which diabetes and hyperlipidemia-induced vascular lesions were created (16,17).

Coronary pathology and experimental hyperlipidemia

Hyperlipidemia was reported to induce atherosclerotic coronary lesions in minipigs as well as in domestic swine (8,18-20). In these studies, hyperlipidemic diet compositions (1% to 6% cholesterol, high proportion of fat, high percentage of saturated fatty acids), duration of feeding and observation periods (six to 50 weeks) varied markedly.

Figure 4) Macrophages. **A** Immunohistochemical labelling of macrophages in a cross-section of the right coronary artery of pig 3 after being fed a 4% cholesterol and 17% coconut fat diet for 13 weeks in combination with streptozotocin therapy (original magnification $\times 40$). **B** Cutout of A. Blue stain indicates macrophages (original magnification $\times 400$)

Only one study using minipigs with a long-term atherogenic diet of 37 weeks and increased LDL cholesterol showed advanced coronary lesions with significant luminal narrowing (21). Interestingly, another study (20) with an observation period of up to 50 weeks (also using minipigs) demonstrated cholesterol-induced accelerated atherosclerosis at certain vascular sites (eg, abdominal aorta and mesenteric arteries) but no significant atherosclerosis in the coronary arteries.

Studies in domestic pigs, with observation periods comparable with our study (up to 13 weeks) and LDL cholesterol levels between 4.6 mmol/L and 9.3 mmol/L (3.3 mmol/L in our study), induced vascular lesions resembling early states of atherosclerotic disease without significant luminal narrowing (8,22,23). Racial and physiological differences between animal models (juvenile domestic pig versus mature minipig) and the varying durations of diet may explain the differences observed in coronary artery histopathology in these studies. In addition, Reitman et al (20) speculated that the regional differences in the extent of atherosclerotic disease at various arterial sites of the same animal could be due to a difference in the susceptibility of arterial sites to the development of atherosclerosis (20). Moreover, the authors hypothesized that there might be a relationship between young age and/or female sex of the minipigs used in their study and the observed lack of significant coronary artery atherosclerosis after a long-term (42 to 50 weeks) high-fat, high-cholesterol diet.

In contrast to other studies, and according to the German law prohibiting ingredients of animal origin in farm animal chow

Figure 5) Pancreatic beta cells. Immunohistochemical staining for insulin. **A** Pig 1 (fed a 2% cholesterol and 17% coconut fat diet, and was not treated with streptozotocin [STZ]) (original magnification $\times 400$). **B** Pig 3 (fed a 4% cholesterol and 17% coconut fat diet in combination with STZ [High Chol+STZ]) (original magnification $\times 400$). **C** Pig 4 (fed High Chol+STZ) (original magnification $\times 400$). Pigs treated with STZ showed no fewer beta cells than the non-STZ treated pigs. White arrows indicate insulin

(transmissible spongiform encephalopathy prevention), we used vegetable fat instead of animal fat (lard or beef tallow). The coconut fat we supplemented to the chow appeared to be particularly suitable because it contains 91% of saturated fatty acids compared with lard (41%) and

beef tallow (54%). Saturated fatty acids increase total cholesterol, whereas unsaturated fatty acids decrease total or LDL cholesterol (8,18,19). This difference in fat compound may explain the slight differences in serum lipid profile compared with other studies in domestic pigs; however, it is very unlikely that this difference had a profound effect on lesion formation.

Coronary pathology and experimental diabetes

Patients with diabetes have a high incidence of CAD and an increased mortality rate due to cardiovascular complications (24), which has supported the effort to develop an appropriate animal model that reflects the main pathophysiological and histomorphological characteristics of human diabetic vascular disease. Because the common vascular characteristics and the interventional possibilities are limited in small animal models compared with in the human setting, the experiences of creating a diabetic model in rodents have been transferred to large-animal models using pigs. Dixon et al (16) showed in a minipig model that after a hyperlipidemic diet was given over 12 weeks, a single alloxan injection (cytotoxic on pancreatic beta cells) induced sustained hyperglycemia and also an increase in fatty streaks as well as functional signs of excess vascular disease (eg, shown in isometric tension studies). Another study (also performed using the minipig model) without a hyperlipidemic diet but with a single STZ injection followed by an oversized bare metal stent implantation 12 weeks thereafter, showed no changes in neointimal area nor in percentage of coronary stenosis in the diabetic versus the nondiabetic group but a higher incidence of stent thrombosis in the diabetic animals four weeks after coronary intervention (25).

Zhang et al (26) investigated the effect of hyperglycemia on coronary arteries three months after a single STZ injection in domestic crossbred pigs. The authors reported increased inflammatory response in the media and adventitia of diabetic animals but no data on histomorphological changes (neointimal area, lumen area stenosis) were presented. In a porcine study of domestic Yorkshire swine, animals were fed a hyperlipidemic diet for up to 48 weeks and 50 mg/kg STZ was injected each day during the first three days of the observation period. The authors reported a significant increase in cholesterol levels during a period of up to 48 weeks and a sustained hyperglycemia during the whole observation period, resulting in severe coronary lesions with significant luminal narrowing of up to 95% stenosis and humanoid features of atherosclerotic coronary arteries such as calcification, necrotic lipid core, etc (6).

Except for the study by Gerrity et al (6), no experimental evidence exists that severe coronary artery lesions with significant luminal narrowing can be induced after a hyperlipidemic diet and/or alloxan- or STZ-induced hyperglycemia in minipig or domestic pig models. In the study by Gerrity et al, domestic pigs had a follow-up period of up to 48 weeks and only the coronary arteries of animals on a long-term hyperlipidemic diet with STZ injection (eg, 20 weeks) showed these significant changes. In contrast, our study and the other studies discussed above had hyperlipidemic diets and follow-ups after alloxan or STZ injection of eight to 13 weeks, different atherogenic diets, and different STZ injection protocols and doses (125 mg/kg to 150 mg/kg). We applied the same STZ injection protocol as Gerrity et al (ie, three injections of STZ on three consecutive days with a total of 150 mg/kg STZ) to avoid life-threatening hypoglycemia caused by a sudden release of insulin due to the massive destruction of beta pancreatic cells as observed in other studies. However, in contrast to the study by Gerrity et al, we did not observe sustained hyperglycemia after STZ injections, nor did we see advanced human atherosclerotic lesions of the coronary arteries. Studies using the minipig model with alloxan injection or split STZ injections showed significant plasma glucose increase but no severe atherosclerotic CAD when analyzed (12,16,27). How can one explain the discrepancy in blood glucose levels after STZ injections between our study and others? Recent evidence suggests that susceptibility to STZ may vary among species. Dufrane et al (28) compared the reactions of domestic crossbred pigs and primates to the

established standard rat model. As far as primates and rats are concerned, a dose of 50 mg/kg STZ induced irreversible diabetes, whereas in domestic pigs with 150 mg/kg STZ, hyperglycemia was reversible despite initial significantly higher blood glucose levels (28). Molecular analysis of these animals demonstrated a low expression of glucose transporter type 2 (GLUT2), which may explain a lower STZ sensitivity in domestic pigs. Moreover, pigs in this study had a higher number of immature beta cells and a compensatory beta cell hypertrophy. Together, these data help in understanding the differences observed after STZ injections between our model and the minipig models mentioned.

For the development of an advanced coronary atherosclerosis model suitable for percutaneous interventional research, it is important to consider that a long-term hyperlipidemic diet, as used by Gerrity et al, will lead to a tremendous weight gain in domestic pigs. Interestingly, none of the studies using atherosclerotic porcine models reported data on weight at the time of sacrifice, although even in minipigs, body size has been considered to be an absolute limitation to their use (29). Our animals had a mean weight of 84 ± 2.4 kg after eight and 13 weeks of the atherogenic diet and STZ injections, which is quite a challenging starting weight for coronary interventional experiments, sometimes requiring another three months of postprocedural observation to investigate late effects. One might speculate on the weight of the domestic pigs in the study by Gerrity et al after a hyperlipidemic diet was given for up to 48 weeks, but it seems unlikely that interventional cardiological and vascular studies will be feasible in these animals.

Limitations

Due to the time-consuming procedures required for the experimental set-up in the current model (eg, anesthesia of the animals for each blood sample drawn), the overall number of animals included in the study was small.

CONCLUSION

The results of our study suggest that it is not feasible to create a model of severe atherosclerotic CAD in a diabetic state in domestic pigs that is suitable for interventional vascular research, using an atherogenic diet in combination with STZ injections. The interspecies differences in STZ response indicate that results of experiments in rodents or in minipigs are not generally applicable to the juvenile, domestic crossbred porcine model. However, in our porcine model, hyperlipidemia induced histomorphological changes in coronary arteries, resembling the initial stages of atherosclerosis but without severe luminal narrowing.

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