



A HIGH CALORIE DIET INDUCES TYPE 2 DIABETES IN THE DESERT SAND RAT (*PSAMMOMYS OBESUS*)

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Abstract – Diabetes mellitus type 2 is a major factor for cardiovascular diseases. The toxic effects of chronic hyperglycemia involve many alterations in the vascular tissue, including atherosclerosis. The pathogenesis of atherosclerosis in the diabetic syndrome (DS-II) has not been fully elucidated. A better understanding of the progress of DS-II at the level of the aorta could help us to identify inhibitors of atherosclerosis. Studies have shown that obesity and high calorie diet (HCD) are associated with the development of DS-II, however the lack of naturally occurring experimental models of DS-II have impaired to directly address these issues. We hypothesize that a HCD induces DS-II. This study (15 months duration) is designed to determine if HCD induces DS-II in the desert sand rat (*Psammomys obesus*; *P. obesus*). We also evaluated the histopathology of the aorta in animals fed with a laboratory chow pellets *ad libitum* (hypercaloric) and in control (animals fed with the naturally occurring hypocaloric diet; halophile plants). The weight and blood chemistry (glucose, lipids, and insulin levels) were evaluated periodically (once per week), and the histology of the aortas of these animals were assessed every 3 months for up to 12 months, during the development of DS-II. This study demonstrates that 40% of the animals in HCD develop DS-II at 3 months. Histological characterization demonstrates the typical alterations observed in atherosclerosis, i.e., alteration of the elastic fibers of the media layer and enrichment in collagen and glucosaminoglycans. This study demonstrates that *P. obesus* is an excellent animal model to study the progression of DS-II and the development of atherosclerosis.

Key words: Angiopathy, diabetes, histopathology, atherosclerosis, *Psammomys obesus*

INTRODUCTION

Diabetes is a major factor for cardiovascular diseases and a known risk factor for atherosclerosis (5-6, 13-14, 17, 20, 24, 28, 38, 41). Cardiovascular diseases, particularly coronary heart disease and peripheral vascular diseases, represent a heavy health and economic burden to society (13-14, 19, 38-39). Evidence for this diabetes-related increase in cardiovascular risk has been reviewed and its relationship to recognized cardiovascular risk factors has been considered (1, 27, 38-41). The causes for the enhanced susceptibility to atherosclerotic disease in diabetes remain controversial.

A better understanding of the progress of type 2 diabetes (DS-II) at the level of the aorta could help us to identify inhibitors of atherosclerosis. Studies have shown that obesity and high calorie diet (HCD) are associated with the development of DS-II (35), however the lack of naturally occurring experimental models of DS-II have impaired to directly address these issues. As experimental model, to study the occurrence of DS-II and atherosclerosis, we used *Psammomys obesus* (*P. obesus*) captured in the wild (2-3, 10, 18, 23). These animals were captured on the sandy beaches of the Nile Delta and are trivially nicknamed 'Sand Rats'.

This study is designed to determine if HCD solely, to the exclusion of all other etiological factors, induces DS-II in the *P. obesus*. We also evaluated the histopathology of

the aorta in control (i.e., animals fed with the naturally occurring hypocaloric diet; halophile plants), and in animals fed a laboratory chow with salty water *ad libitum* (hypercaloric diet: HCD). The weight and blood chemistry (glucose, lipids, and insulin levels) were evaluated periodically (once per week), and the histology of the aorta of these animals was evaluated every 3 months for up to 12-15 months, during the development of DS-II. This study demonstrates that 40% of the animals in HCD develop DS-II at 3 months. At 6 months, 70 % of these DS-II animals develop insulin dependent diabetes. The litters from animals exposed to HCD all develop diabetes at birth. Histological characterization demonstrates the typical alterations observed in atherosclerosis. This study demonstrates that *P. obesus* is an excellent animal model to study the progression of DS-II and the development of atherosclerosis.

MATERIALS AND METHODS

Animals

Psammomys obesus (*P. obesus*) are highly specialized members of the *Muridae* family and *Gerbillidae* subfamily. They originate from the dry- and semi-barren backgrounds of North Africa. There are three species called "Sand rat": *P. obesus* of the Algerian south west (herein referred as 'natural' *P. obesus*), Tunisian *P. obesus* and *Psammomys vexillaris*. Another well known inbreed model called 'Israelis Sand Rat', originally captured from the north desert area of the Dead Sea were obtained from Harlan (Hebrew University Animal Facility, Israel), where the 'Diabetes Prone' and Diabetes Resistant' lines are generated by assorted breeding (21-22, 35).

Psammomys obesus (*P. obesus*), used in this study, were captured by the National Research Centre (CNRZA; Béni-Abbès) on arid areas in the south-west Algerian Sahara (30°7 north latitude, 2°10 west longitude; Bechar). Their natural diet is essentially plants with a high water and salt content, including: *Chenopodiaceae* family, *Traganum nudatum*, *Salsola foetida* and *Suaedia mollis* species). At their reception, the animals were placed in the vivarium under well controlled temperature and hygrometry. The conditions of captivity in the laboratory are kept near to their natural biotope (temperature: 25 °C hygrometric degree: 60 %). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* [DHHS Publ. No. (NIH) 85-23, revised 1996, Office of Science and Health Reports, Bethesda, MD 20892].

We used 62 mature (3-6 months) *P. obesus* of both sex divided in 2 groups. 24 controls were fed with the naturally occurring hypocaloric diet (50 g/day which is equivalent to 20-22 cal/day) and 38 treated animals (15 males and 23 females) were fed with laboratory chow pellets (hypercaloric diet; 10 g/day which is equivalent to 32.5 cal/day and salty water, NaCl 0.9 %, *ad libitum*). The NaCl quantity used in this study is low and does not trigger hypertension (31-32)

The hypocaloric diet is based on halophile plants (*Suaeda mollis*). Table 1 shows the composition of the diets. The weight, blood and urinary samples were monitored once a week.

Blood samples were collected from the retro-orbital sinus with a heparinized capillary tube. The plasma was obtained after centrifugation. To determine the glucose levels, plasma samples were deproteinized and glucose levels were determined by the Somogyi-Nelson procedure (29, 37). The lipids were extracted from the plasma by the method of Folch and co-workers (12) and quantified gravimetrically with an electrobalance. The lipids were separated on thin, silica-coated quartz rods (chromarod S) equipped with a TLC flame ionization detector (Iatroscan). Since *P. obesus* has oliguria it was not possible to use the routine quantitative method. Therefore, to determine glucosuria, ketonuria and albuminuria, urine samples were analyzed by test strips analyser, a qualitative method that provides clinically significant data.

The plasma glucose, insulin, lipids; and glucosuria, ketonuria and albuminuria levels are shown in table 2.

Histochemistry

Animals were sacrificed at three months intervals for up to 15 months. The upper thoracic aorta was dissected and cut into small pieces of about 1 mm³. The tissues were immediately fixed in 2 % glutaraldehyde for 3 hours at ambient temperature. Then, the samples were rinsed with a buffer washing solution SORESENSE (4 successive baths for 1 hour) and then incubated overnight in the last bath. Thereafter, the samples were incubated in 1% osmium tetroxide (OsO₄-HgCl₂) for 1 hour (ambient temperature), and then rinsed with distilled water.

The samples were dehydrated by successively increasing concentrations of ethanol : 50° (5 min); ethanol + 1.5% uranyl acetate (1 hour in dark); ethanol at 75° and 95° (5 min each); three baths of ethanol at 100° (10 min each) and two baths of propylene oxide (10 min each). Samples were then embedded in a synthetic resin marks Epon (40.5 ml of Epikote Epon 812 + 22.5 ml of Nadic methyl anhydre + 25 ml of Dodecyl succinic anhydride) as follows: 1 hour bath in an equal parts mixture of propylene oxide and Epon followed by a bath of 12-18 hours in Epon + DMP 30 mixture and by an inclusion in gelatine capsules.

The polymerisation was obtained after 48 hours in a steam room at 60°C. The tissue sections were obtained with an ultra microtome L.K.B (8800 Ultratome III) which allowed to obtain ultra thin sections (60 nm). The sections were collected on copper grid with no film support. The sections were imbedded lightly in uranyl acetate and lead citrate according to Reynolds (30). The tissue samples were analysed with a RCA EMU-3G electron microscope (Radio Corp. of America, Camden, N.J).

Statistical analysis

Values are the mean ± SE. Statistical analysis (insulin, phospholipids cholesterol and triglycerides) was performed using a two-way ANOVA with Tukey's test for multiple comparisons (Graph pad statistical software). *P* < 0.05 was considered statistically significant.

Table 1. Hypocaloric and hypercaloric diets Composition

	Hypocaloric Diet Composition¹ (percentage)	Hypercaloric Diet Composition² (percentage)
H ₂ O	80.79	9
Minerals	6.86	7.1
Fats	0.40	7.5
Proteins	3.53	25
Total sugar	0.18	47.4 ³
Lignine	1.12	-
Hemicellulose	2.62	-
indetermined	2.27	-
Cellulose	2.23	4

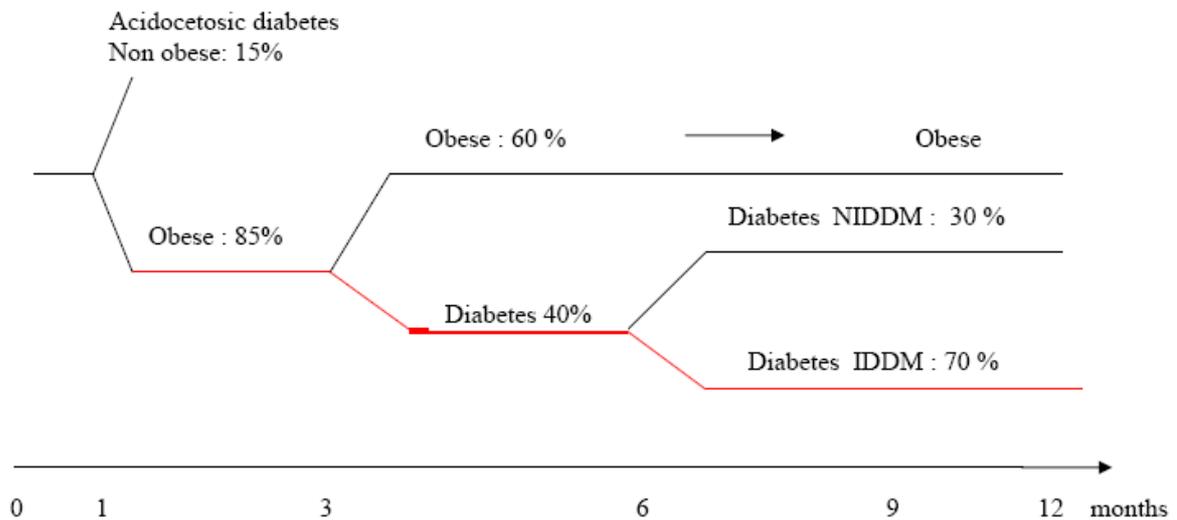
¹The energetic value is estimated at 450 calories/kg. The animals were fed 50 g/day, representing 20-22 cal/day.

²The energetic value is estimated at 3250 calories/kg. The animals were fed 10 g/day, equivalent to 32.5 cal/day.

³Rate of usable glucids (= 100 - (rate of raw proteins + rate of raw fat matters + rate of raw ashes + rate of raw cellulose + rate of humidity).

Remark: the carbohydrates (total sugars, lignine, hemicelluloses, cellulose and indetermined quantity) constitute 8.42% of the total composition.

Diagram 1. Representation of the behaviour of the *P.obesus* in HCD during the development of the obesity and diabetes.



RESULTS

Animals

In its natural environment *P. obesus* never develops neither obesity nor hyperglycemia or hyperinsulinemia. However, *P. obesus* maintained in the laboratory and fed with

a standard laboratory diet (HCD) during 12-15 months, developed a number of metabolic disorders associated with obesity and diabetes (diagram 1). We observed that 15 % of the HCD animals do not survive more than 3-4 weeks. These animals developed a significant decrease in glucose tolerance with low plasma

Table 2. Body Weight, Blood and Urine Metabolic Parameters in *P. obesus* during HCD diet.

Time (months) group	Weight (gr)	Glycaemia mg/100ml	Urinary tests	Insulin μ U/ml	Phospholipids mg/100ml	Cholesterol mg/100ml	Triglycerides mg/100ml
0	74 \pm 2	64.5 \pm 1.8	G =K=A= 0	25 \pm 1.8	65 \pm 1.3	49 \pm 2.8	76 \pm 3.8
3	85 \pm 2.4	63 \pm 1.8	G =K=A= 0	27.2 \pm 2.3	64 \pm 2	55.4 \pm 2.5	70.7 \pm 3.8
3 Obese	98.8 \pm 2.0	79.8 \pm 2.9	G = 0 K = 0 A = 0	140.2 \pm 0.4	75.2 \pm 2.92	77.8 \pm 2.77	97 \pm 7.48
3 Diabetic	105 \pm 3.1	181 \pm 18	G = 0 to ++ K = 0 to + A = 0 to +	265 \pm 21	74 \pm 15	119 \pm 11	124 \pm 24.7
6	99.8 \pm 3.3	64.7 \pm 2	G = K= 0 A = 0 to +	30.6 \pm 2.0	67 \pm 2.35	60.0 \pm 2.9	75.5 \pm 5.8
6 NIDDM	111 \pm 3	171 \pm 21	G = 0 to ++ K = 0 to + A = 0 to +	309 \pm 27	84 \pm 2.4	120 \pm 12	230 \pm 22 *
6 IDDM	87.3 \pm 7	407 \pm 35 *	G = ++ to +++ K = ++ to +++ A = 0 to ++	133 \pm 14 *	101 \pm 4 *	134 \pm 7	377 \pm 53 *
9	106 \pm 3	69.5 \pm 2.4	G = K = 0 A = 0 to +	33 \pm 2.5 *	71 \pm 3	59.5 \pm 3.0	81.0 \pm 4.7
9 NIDDM	118 \pm 7	197 \pm 25 *	G = 0 to ++ K = 0 to + A = 0 to ++	384 \pm 43 *	85 \pm 2	124 \pm 10 *	252 \pm 27 *
9 IDDM+I.	80.7 \pm 3 *	428 \pm 26 *	G = + to +++ K = + to ++ A = 0 to ++	141 \pm 19 *	114 \pm 7 *	202 \pm 12 *	676 \pm 103 *
12-15	106 \pm 2	66 \pm 2	G =K= 0 A = 0 to +	35 \pm 2.5	68 \pm 2	71 \pm 3	87 \pm 6
12 NIDDM	129 \pm 5 *	221 \pm 31*	G = 0 to +++ K = 0 to + A = 0 to ++	438 \pm 44 *	88 \pm 2*	134 \pm 11*	306 \pm 34 *
12 IDDM+I.	82 \pm 3 *	399 \pm 20 *	G = ++ to +++ K = + to +++ A = + to ++	175 \pm 47 *	136 \pm 12 *	215 \pm 27 *	826 \pm 115 *
15 NIDDM	143 \pm 7 *	240 \pm 38 *	G = 0 to +++ K = 0 to + A = 0 to ++	502 \pm 47 *	105 \pm 9	147 \pm 15 *	373 \pm 50 *
15 IDDM+I.	78.5 \pm 2 *	421 \pm 27 *	G = + to +++ K = + to +++ A = + to ++	217 \pm 49	162 \pm 10 *	300 \pm 39 *	1071 \pm 101 *

G : glucose ; K : ketonuria, ; A : albuminuria

I. : the animals were treated with insulin in an intermittent manner in order to avoid the diabetic coma and the death of the animal.

* P<0.05 compared from their respective control group (O, 3,6,9, 12-15) Data are means \pm SE for 5 to 12 animals per group

insulin levels, followed by death after severe acidoketosis. All the remaining (85 %) animals developed obesity and elevated plasma insulin levels.

The weight, blood and urinary chemistry (glucose, lipids, and insulin levels) determined once a week for up to 15 months, are shown in table II. Up to 3 months in HCD, 40 % of the animals developed DS-II followed by hyperinsulinemia, hyperglycemia, decreased dependent diabetes were treated with insulin injection.

We noticed also that the litters from these animals exposed to HCD all developed diabetes at birth. Importantly, animals kept in the laboratory and fed with their naturally occurring diet (halophile plants, low in calories) did not develop diabetes. Their litters did not develop diabetes either. These data indicate that diet determines the development of DS-II in *P. obesus* fed with HCD (table 2).

Electro-Microscopy Studies

Morphology and Ultrastructure

The study of morphology and ultrafine sections reveals the structure of the three principal zones in the artery: the intima, media and the adventitia.

Healthy artery in control animals

The morphology of the normal artery of young and adult animals is shown in figures 1A-1B.

The ultrastructure of the intima (figure 2A) is formed of one hardcore of elongated, flatten cells, slightly rounded at the core level. The core is lobed, flatten. The chromatin is dense, most of the time attached to the nuclear membrane. We noticed a few mitochondria. The sub endothelial zone is formed of a space limited by the endothelium and the internal elastic lamina, which is continuous, thick, with fair womb where lengthwise fibrillation are sometimes detectable. The endothelium is separated from the internal elastic lamina composed by collagen fibres.

The media is the most substantial tunic of the artery. It is made up of smooth muscle cells layers interspersed with elastic strips and collagen fibres. The smooth muscle cells are very elongated (figures 3A-B). Their cytoplasm is dense and non-vacuolated, containing abundant bundles of myofilaments. The ergastoplasm is very abundant.

glucose tolerance, and increased to insulin resistance. By the end of six months, we observed, in 70 % of the diabetic animals, a significant decrease in the body weight associated with a decrease in the plasma insulin levels and an increase of glycemia, glycosuria and ketonuria. The plasma triglyceride content and the lipid deposits in the liver were also increased (data not shown). Animals with insulin

The adventitia (figure 4A) is the most external, protective layer, made up of longitudinally arranged collagen fibres and some elastic elements. We observed large blood vessels or 'vasa-vasorum' and afferent nerve fibres. This layer, mainly made of connective tissue, exhibits a few cells.

Altered artery in obese animals

After three months in HCD, the changes were essentially observed in the media layer. These changes were characterized by thinning and rupture of the elastic strips. Figures 3 C-D show alterations in the connective tissue, abundance of collagen in this layer, and numerous cells with clear cytoplasm. At 6 months in HCD and thereafter, the elastic strips of the media became much more disorganized. The collagen was very abundant and there were diverse cellular changes in the internal half of the media layer. However, no changes were observed in the intima or the adventitia layers (figures 4B-C).

Altered artery in diabetic animals

In non-insulin-dependent diabetic animals, the changes were essentially observed in the tunic media. From 3 to 6 months, we observed severe conjunctive and cellular lesions (figures 5A-B).

In insulin-dependent diabetic animals, the changes in the media were more severe at 6 months than at earlier time points. The connective tissue is disorganized (figure 5C). The elastic strips were thinned and frayed. The smooth muscle cells are invaded by the collagen that creates a real fibrosis. These cells exhibit hypertrophy with a very clear cytoplasm. From 6 to 12-15 months, the observed changes are more dramatic. An important degeneration of the media elastic strips as well as of the internal elastic lamina was observed. A development of collagen with accentuation of fibrosis and denaturation of the smooth muscle cells, that

entails a real necrosis, was also apparent (figure 5D).

The ultrastructural analysis for the artery of the diabetic animals confirmed that the arterial disorders mainly concern the smooth muscle cells and the connective tissue of the tunica media. The early appearances of smooth muscle cell's distortion (three months) corresponded to the appearance of 'fair' cells with little dense cytoplasm, where myofilaments have virtually disappeared (figure 1C). The ergastoplasm was more developed, but in the whole these dedifferentiated cells were poor in organelles (figure 1D). These smooth muscle cells lose their interspatial links and separate within the artery. We observed some giant cells characterized by numerous mitochondria and a very lobed core (figure 1E). The affected cells in the media layer exhibit mitochondrial alterations (from swelling of the crest womb to denaturation of their matrix), significant vacuolization, generalized

clarification of its cytoplasm, and a denaturation of the nuclear matter.

From 9 to 12-15 months, the smooth muscle cells in the media layer were completely necrotic (figure 1F).

Figures 2 B-D show the aorta in the insulin-dependent diabetes mellitus state. The strips of the elastic framework and the internal elastic lamina were particularly affected. The strips were slimmed and their diameter was irregular. The elastic tissue was mainly made up of coalescent or scattered nodules between the cells in the media zone. The arterial structural integrity was disrupted since the collagen became very abundant and filled entirely the spaces between the elastic elements and the cell layers. This excessive collagen formation is possibly due to de-differentiation of the smooth muscle cells that actively synthesize the precursor of collagen.

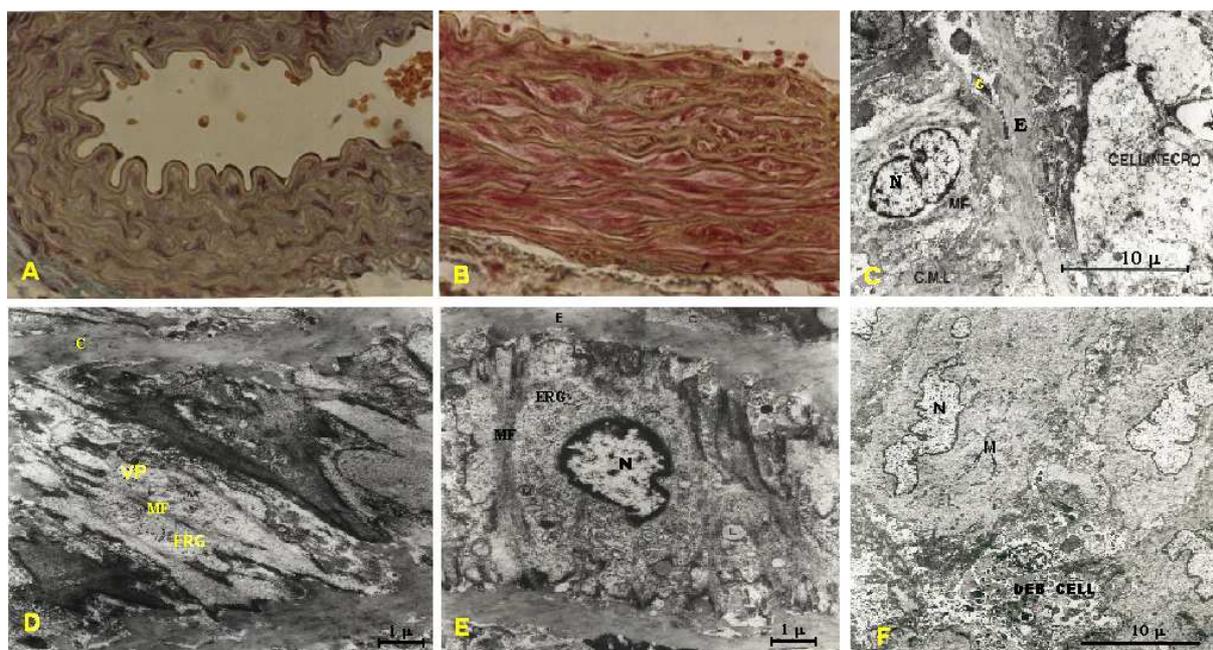


Figure 1. A-B: Morphology of the healthy artery from non-diabetic: young (A) and adult (B) animals. (x400). C-F: Ultrastructure of the altered artery from diabetic animals. (C) Necrosis of the smooth muscle cells. (x9000). (D) Ultrastructural abnormalities of the smooth muscle cells. (E) dedifferentiation of the smooth muscle cells; development of the ergastoplasm, disappearance of the myofilaments. (F) 'giants' cells, atypical cells and cellular debris. (x 16000). DEB.CELL: cellular debris. C : collagen. CELL.NECR: necrosed cell. C.M.L : smooth muscle cell. E : elastin. ERG: ergastoplasm. L : lipids. M : mitochondria. MF : myofilament. N : nucleus. VP : pinocytosis vesicle.

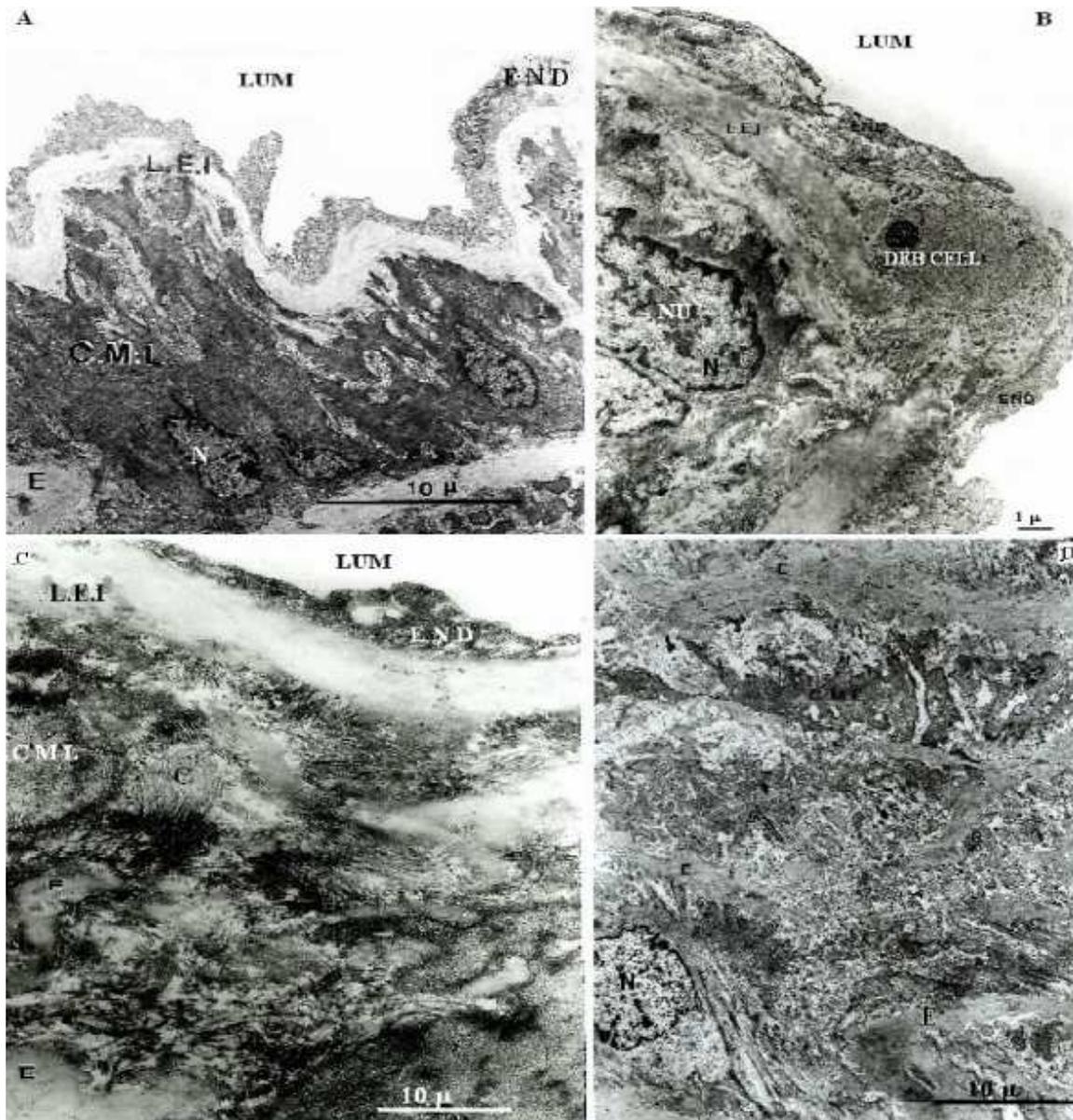


Figure 2. Morphology of the healthy artery. (A) Ultrastructure of the intima. (x4000)

Morphology of the altered artery from diabetic animals. (B) lesions of the intima, disjunction of the endothelial cells, rupture of the internal elastic lamina, under endothelium cellular debris. (x 12500). **(C)** degeneration of the elastic structures (non coalescent nodules) and proliferation of the collagen tissue. (x 25000). **(D)** degeneration of the media, numerous ruptures of the elastic strips, cellular alterations, very abundant collagen. (x 5500). C: collagen. C.M.L: smooth muscle cell. DEB.CELL: cellular debris. E: elastin. END : endothelium. L.E.I.: internal elastic lamina. LUM: lumen of the artery. N: nucleus. NU: nucleoli

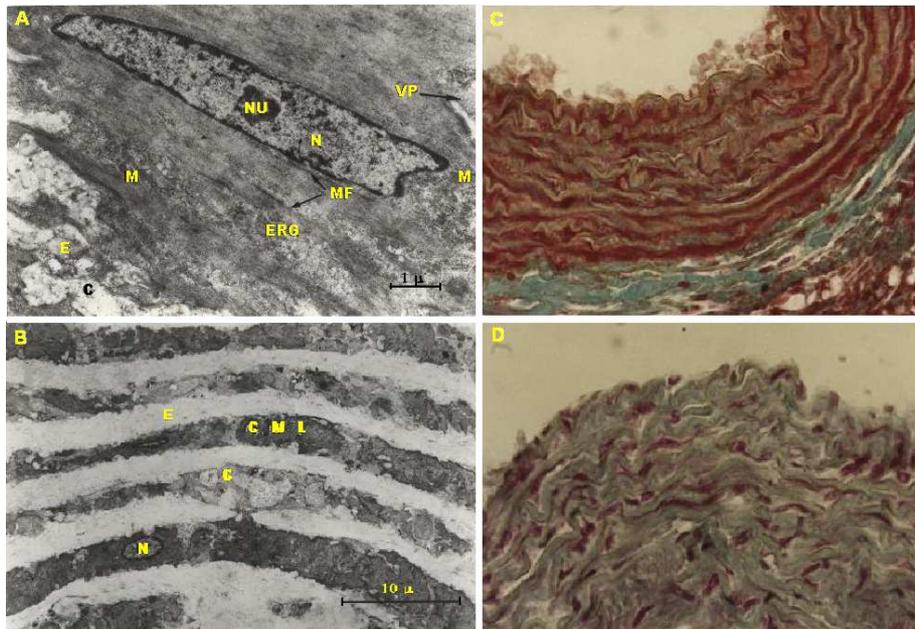


Figure 3. (A) and (B). Ultrastructure of the media of the healthy artery. (A) medial smooth muscular cells. (x18 000); **(B)** media (x4000). **(C) and (D) Histology of the media of the obese artery at 3 months. Ultrastructure of the media (C)** alteration of the elastic tissue. (x400); **(D)** alteration of the elastic tissue, abundance of collagen. (x400). C: collagen. C.M.L.: smooth muscle cell. E: elastin. ERG: ergastoplasm. M: mitochondria. MF: myofilament. N: nucleus. NU: nucleoli. VP: pinocytosis vesicle.

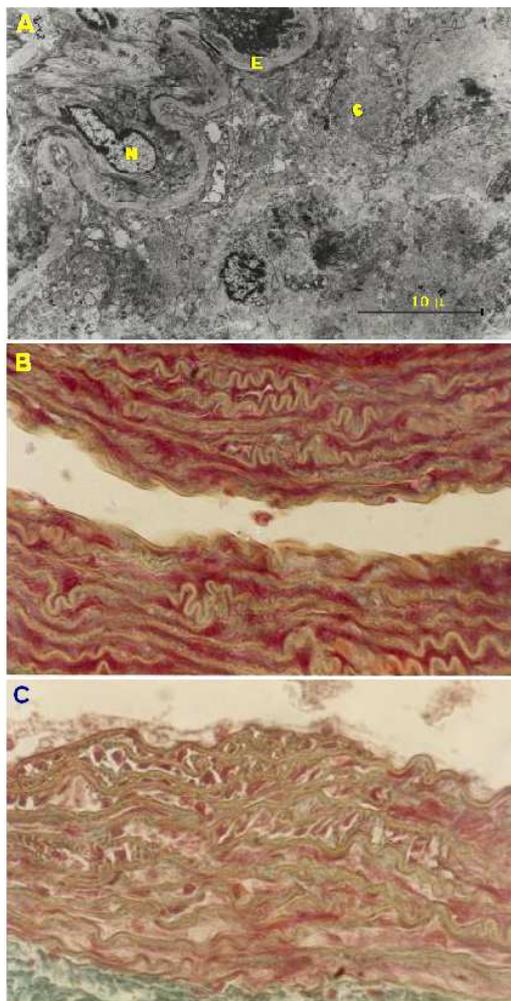


Figure 4. (A) Ultrastructure of the adventitia of the healthy artery. (x4000) **(B) and (C) Histology of the obese artery. (B)** Severe injuries in the elastic tissue, abundance of collagen at 6 months. **(C)** Severe injuries in the connective tissue and cellular lesions. (x400). C: collagen. E: elastin. N: nucleus.

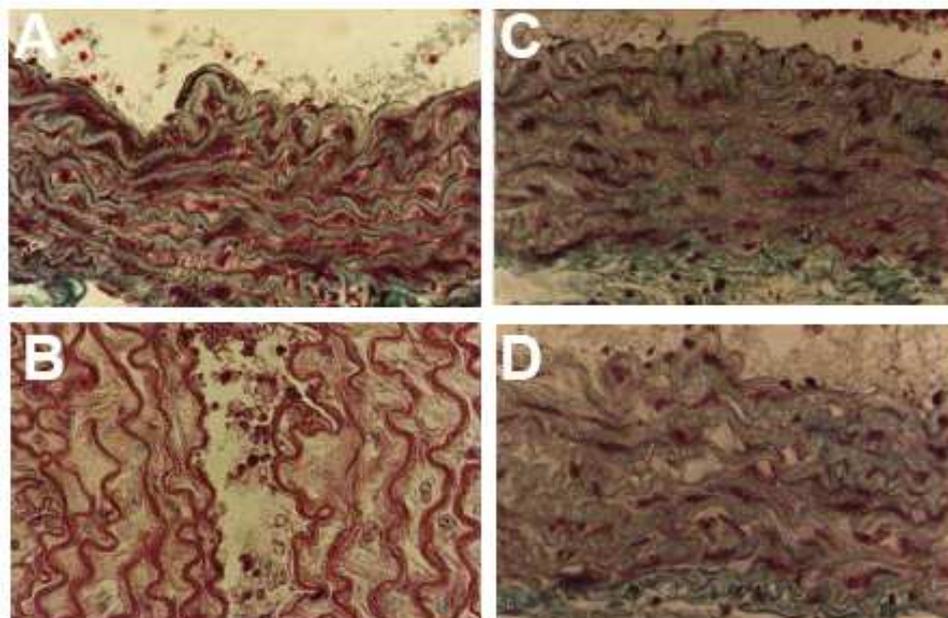


Figure 5. (A) and (B) Histology of the artery from non-insulin-dependent diabetic animals. (A) Alteration of the conjunctive tissue and some cellular lesions at 3 months. (B) Severe conjunctive and cellular lesions at 6 months. (x400). (C) and (D) Histology of the artery from insulin-dependent diabetic animals. (C) serious conjunctive and cellular lesions at 6 months. (D) Severe conjunctive lesions and cellular necrosis at 9 months. (x400).

DISCUSSION

The aim of this study was to examine the long-term effects of a standard synthetic-chow laboratory diet which represents a High Calorie Diet for the sand rats, on the metabolic pattern of the diabetic syndrome in the sand rat, *P. obesus*. Our study shows that the ultrastructural alterations in the aortic tunic media of *P. obesus* are similar in either obese, normoglycemic (with or without hyperinsulinemia), or in animals where hyperinsulinemia is accompanied with hyperglycemia leading to IDDM. However, the frequency and magnitude of these ultrastructural alterations are dependent on the metabolic status of the animal.

Numerous experimental models have been used to study atherosclerosis: the obese mouse (ob/ob), the New Zealand obese mouse, the Japanese mouse K.K, the Chinese Hamster C.G, the fed up mouse fa/fa, the diabetic mouse db/db, and the spontaneously diabetic wistar rat BB (4, 7-8, 17, 25, 27). However, all these animal models are not always representative of the atherosclerosis observed in human disease, since these models develop only trifling vascular lesions that are limited to the level of the lipidic strips without vascular lesions affecting the arterial trunk. Unlike most mouse strains, which

are highly resistant to atherosclerosis, the apoE-KO mouse strain develops atherosclerotic lesions in a distribution closely resembling human disease (16, 26). The induced-diabetes models (i.e., streptozotocine) also develop molecular alterations and lesions resembling to those described in human (11, 34). However, these diabetic models show little information on the vascular pathology related to diabetes. Therefore, the *P. obesus* used in this study allow us to study naturally occurring DS-II and the development of atherosclerosis (46-48). Our study shows the histological alterations and the natural history of diabetes type II development in the sand rat submitted to high calorie diets (HCD). Our study also allow us to follow the natural progression of obesity coupled to high calorie diets with the development of DS-II (36, 42-43). Moreover we have shown that the natural *P. obesus* of the southern west of Algeria has a nutritional syndrome characterized by a relative caloric overload, similar to what is observed in patients and to Israelis Sand Rat, (21, 35, 42-48).

The most significant alterations are typical of animals in the last stage of diabetes (IDDM), where there are frequent and more critical arterial alterations, including necrosis. The apoptosis of the beta cells during the hyperglycemia phase in *P. obesus* has been confirmed by in vitro studies (9).

Finally, the obese animals present also cellular and connective tissue deteriorations that develop progressively and that are apparent at the third month in HCD diet. However, in the sixth month they are less important than that observed among hyperglycemic or IDDM animals. The media layer of the *P. obesus* in hyperglycemia or in hyperinsulinemia state (a characteristic feature of NIDDM) is also affected albeit at a lower degree. In particular, areas of cellular necrosis are scarce. Thus, it seems that the importance of the observed arterial alterations vary according to the seriousness and the length of the diabetic disease.

In old atherosclerotic rodents, disorders of the same nature as those described in non-aged *P. obesus* have also been observed. The morphological, histochemical and histoenzymological studies performed during the ontogenesis of the aorta have shown an atherosclerosis predisposition of the *P. obesus* (2, 3, 10, 23). Therefore, we suggest that the 'natural' sand rat *P. obesus* represents an excellent model to study atherosclerosis in association with diabetes in non-aged rodents.

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