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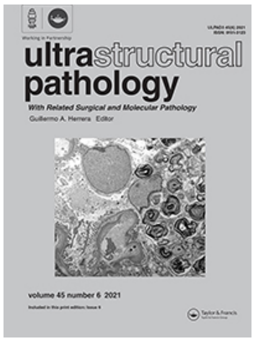

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BASIC RESEARCH

Leptin receptor defect with diabetes causes skeletal muscle atrophy in female obese Zucker rats where peculiar depots networked with mitochondrial damages

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ABSTRACT

Tibialis anterior muscles of 45-week-old female obese Zucker rats with defective leptin receptor and non-insulin dependent diabetes mellitus (NIDDM) showed a significative atrophy compared to lean muscles, based on histochemical-stained section's measurements in the sequence: oxidative slow twitch (SO, type I) < oxidative fast twitch (FOG, type IIa) < fast glycolytic (FG, type IIb). Both oxidative fiber's outskirts resembled 'ragged' fibers and, in these zones, ultrastructure revealed small clusters of endoplasm-like reticulum filled with unidentified electron contrasted compounds, contiguous and continuous with adjacent mitochondria envelope. The linings appeared crenated stabbed by circular patterns resembling those found of ceramides. The same fibers contained scattered degraded mitochondria that tethered electron contrasted droplets favoring larger depots while mitoptosis were widespread in FG fibers. Based on other interdisciplinary investigations on the lipid depots of diabetes 2 muscles made us to propose these accumulated contrasted contents to be made of peculiar lipids, including acyl-ceramides, as those were only found while diabetes 2 progresses in aging obese rats. These could interfere in NIDDM with mitochondrial oxidative energetic demands and muscle functions.

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KEYWORDS
Diabetes 2; skeletal muscle; atrophy; lipids; mitochondria; ceramide

Introduction

> We must always tell what we see. Above all, and this is more difficult, we must always see what we see. Charles Péguy (1873–1914).

Diabetes is a worldwide-distributed metabolic malady that afflict people with type 2 or non-insulin dependent diabetes mellitus (NIDDM), typically developed in aging adults. Nowadays, the rate of diabetes 2 is also increasing in all ages, including children and young adults, due to overweight, unhealthy diet and physical inactivity. Diabetes 2 has been known since Antiquity[1] and the topic has been reviewed by an immense number of clinical care specialists in biomedical fields. Its impact on public health cost is surveyed by national and international organizations of medicine, because its metabolic alterations favors many other disabilities and pathologies leading to an excess of fatalities before age 70.[2–9] One of the etiologies is a defective adipokine leptin receptor.[10–12] The animal model that best matches human leptin receptor defect is the genetically obese Zucker rat[13–33] which progresses at an early age to diabetes 2 because, soon after weaning, young male and female rodents of the *fa/fa* (obese) strain manifest hyperphagia.[12] Thus, at young age, this rodent rapidly develops a clear phenotypic obesity due to leptin excess with hyperinsulinemia and insulin insensitivity. Consequently, these growing and aging rats undergo other endocrine entwined defects that favored multiple organ

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*JG and WRP dedicate this study to the late Dr F Norman Paradise, colleague, and colleague and mentor at the Northeastern Ohio Universities College of Medicine (now NEOMed), USA and to Dr Albert Claude, 1974 Nobel laureate, who reviewed JG doctoral thesis on muscle ultrastructure.

function's changes similarly to what one can find in most of the clinical progression in the human NIDDM in diabetes type 2.[13–33]

Even though the skeletal muscles encompass about 40% body weight and the tissue plays an important regulatory function in expenditure due to its functions in locomotion and metabolism,[34] it is only a very small number of ultrastructure reports that have been published about the human obesity and diabetes 2 skeletal muscles.[35–37] There, lipid depots but mitochondria functions seemed to have lastly delved on this last organelle in NIDDM [e. g. [37–46] but this focus topic is not without controversy.[47] Like in human diabetes 2, the obese Zucker rat skeletal muscle histopathology does not appeared strikingly changed from a normal muscle sample with light microscopy and, thus, has remained neglected insofar about its fine features with aging. These and other tissues would be also influenced by several defective leptin transduction signals, including endocrine secretions out of hypothalamus and peripheral tissues (e.g. ghrelin in the stomach lining; adiponectin, resistin from adipose tissues), [10,11,23–27] as well as of the thyroid glands.[29–31] Muscle tissues are potent targets for the iodinated hormones to stimulate mitochondrial metabolic expenditure and, thus, could provide some relief for diabetes progress through increased storage's anabolism[38,48–54] in addition to or accompanying recent medications,[55] including in this Zucker rat model.[56]

This report extends an early histochemistry study, complemented by some preliminary electron microscopy investigations as abstracts.[57,58] Altogether, our fine structure data further show that diabetes 2 accompanied by leptin receptor defect, induces some skeletal muscles (in this case, the tibialis anterior muscles) of old female diabetic rats to atrophy also caused by a progress in their defective innervation.[32,33] Moreover, the oxidative fibers cursory examination of its semi-thin sections appeared with ragged aspect and the fine structure of these revealed undescribed electron-contrasted interconnected depots, liposome-like components of endoplasmic contiguities and continuities with adjacent mitochondria outer membranes. Based on recent literature and other in vitro data, we can point out that those stored lipids and other electron contrasted components could include ceramides and metabolites, key impeding compounds of insulin and leptin sensitivity.[59–63] Yet, at the time of these investigations, a lack of funding and time made us not able to further identify and characterize these depots by markers and complementary techniques because the same oxidative fibers showed scattered damaged and lytic mitochondria as remnants out of 'mitoptosis,' instead of mitophagy.[45–47,64] Interestingly, some of the damaged organelles appeared to house or accumulate similar, unidentified electron contrasted materials and lipids. Finally, the highest number of mitolyses, including mitoptosis, without involving lipid-like content were revealed in the fast glycolytic (FG) fibers. Associated with NIDDM, would these organelle's eliminations be part of ambulatory weakness due to FG fibers developing defects with time, as in human? .[65]

Material and methods

Ethical concerns

The Institutional Animal Care and Use Committee (AAALAC) of the Northeastern Ohio Universities College of Medicine (NEOMed), Rootstown, Ohio approved all the experimental protocols (animal maintenance, experimentation, anesthesia, sacrifice and/or euthanasia procedures) of the Zucker rats by Dr J Finkelstein who used them for brain studies,[23,24] endocrine organs and peripheral nerves[29–33] and by Dr N F Paradise for cardiac functions.[61,62] We were allowed to also use the rat's remains to excise several other organs, including the tibialis anterior muscles, used for this investigation.

Animal care and tissue's collection

The obese Zucker (or fatty) female rats that have both recessive traits (*fa/fa*) while Zucker rats *Fa/?* were the lean rats. Both genotypes possible of lean Zucker rats (either *Fa/Fa* or *Fa/?*) due to their either dominant homozygous trait or heterozygous; there, mark '?' indicates the uncertain trait associated with the lean rat used in laboratory, as relying on its morphology, characteristic of 'lean' or at least heterozygous rats as noted in previous publications.[29–33]

Five female obese Zucker rats (fa/fa) (45 weeks of age, 584 ± 20.2 g) and five lean littermates (Fa/?) (271 ± 11.5 g) out of a colony of rats purchased from Charles River Laboratories (Raleigh, NC) derived from original stocks[13–15,21,23,24] were all maintained in a constant environment (22°C) with a reversed 12 h/12 h light/dark cycle because the same age groups were part of another experiment dealing with exercise. The cycle was reversed to facilitate better running performance as rats are nocturnal animals. Purine lab chow and water were available ad libitum throughout their care.

Light (LM) and transmission electron (TEM) microscopy

Histochemistry

While hearts were used for cardiac performance investigations[66,67] and necessitated fast dissections avoiding interfering anesthesia, these 45-week-old female rats, were sacrificed by decapitation. Three tibialis anterior muscles from lean and from obese female Zucker rats were excised for histochemistry, frozen by isopentane cooled in liquid nitrogen, and 10-μm serially cut sections at −25°C were incubated for Ca^{2+}-activated ATPase (E.C. 3.6.1.3) by the method of Guth and Samaha (pre-incubation at pH 10.4)[68] and for succinate dehydrogenase (E.C. 1.3.99.1) or SDH[69] as applied in other skeletal muscle investigations.[70–72] Reagents were obtained from Sigma Chemical Co (St Louis Mo). Measurements of muscle diameters were accomplished by measuring the widest diameter of each fiber profile in one direction, then at right angle to the first, and taking the average of both values using Song's technique[73] and an Apple morphometric program.[70–75]

TEM processing

The rat's corpses used for LM were rapidly perfused with 3.5% buffered glutaraldehyde solution (0.1 M Na cacodylate, pH 7.35, at room temperature for 15 min), as in[71] and the 3 contralateral legs, sectioned with tibialis muscles still in situ, were excised to undergo the same fixation that continued for 2 h at 4°C. Washed in buffered sucrose solution, segments of muscle specimens were thinned into muscle fiber bundles, postfixed in 1.5% aqueous osmium tetroxide solution and processed for transmission (TEM) electron microscopy after embedment in PolyBed epoxy resin (Polysciences, Warrington PA.). One-μm thick sections, stained by toluidine blue, were observed with an Olympus BX51 light photomicroscope (Olympus America, Melville NY) to select areas for ultramicrotomy. Ultrathin sections were collected on 50, 75- and 100-mesh hexagonal copper grids (SPI, West Chester PA), contrasted by uranyl acetate and lead citrate prior to be examined in a JEOL 100 S electron microscope (JEOL USA, Inc, Peabody, MA).

Statistical analyses

Statistical analyses were performed with GraphPad Prism (v 7.0) statistical software. Normal distribution of fiber size was evaluated using a Kolmogorov-Smirnov test. All data were expressed as means ± s.e.m. Two-tailed Mann-Whitney test was used to test for differences between lean (fa/fa) and obese (Fa/?) rats, with a significant difference set at $p< .05$. One-way Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test was applied to compare between both rat's 3 fiber types (FG, FOG and SO). Similar quantitative comparisons have been done concerning mitochondrial damages and lipid depots in relationships with section's areas observed.

Results

Light microscopy (LM)

General histology

The LM surveys of the semi-thin sections, stained by toluidine blue, the muscle fiber profiles displayed diverse aspects of staining characteristics, allowing to recognized them as 3 main types, with their specific staining topography. A brief qualitative survey allowed to recognize that the whitish-stained were always the widest fiber profiles, likely being the fast glycolytic fibers, displaying an almost transparent orthochromatic aspect compared with oxidative fibers that were narrower than the first ones. Moreover, the strongest with toluidine stain ones were the thinnest, matching the SO type with histochemistry (see 1.b) and all FOG revealed outskirts whose qualitative profiles revealed many longitudinally-oriented, elongated, narrow inter-myofibrillar masses and thick subsarcolemmal (and perikaryal) accumulations of admixed orthochromatic and metachromatic contrast. This morphology aspects made the fibers to appear more of less serrated, seemingly 'ragged' according to the randomness plane of thin sectioning. All semi-thin sections revealed their fine muscle cross-striations. The endomysium, made of intercellular loose connective elements, is displayed as narrow gaps between muscle fibers where small blood vessels, mainly capillaries, can be revealed. (Figure 1(a-d)).

Histochemistry and morphometry

Following samples of obese and lean muscles stained by classic histochemical markers as fiber types, a cursory look made LM aspects of all the

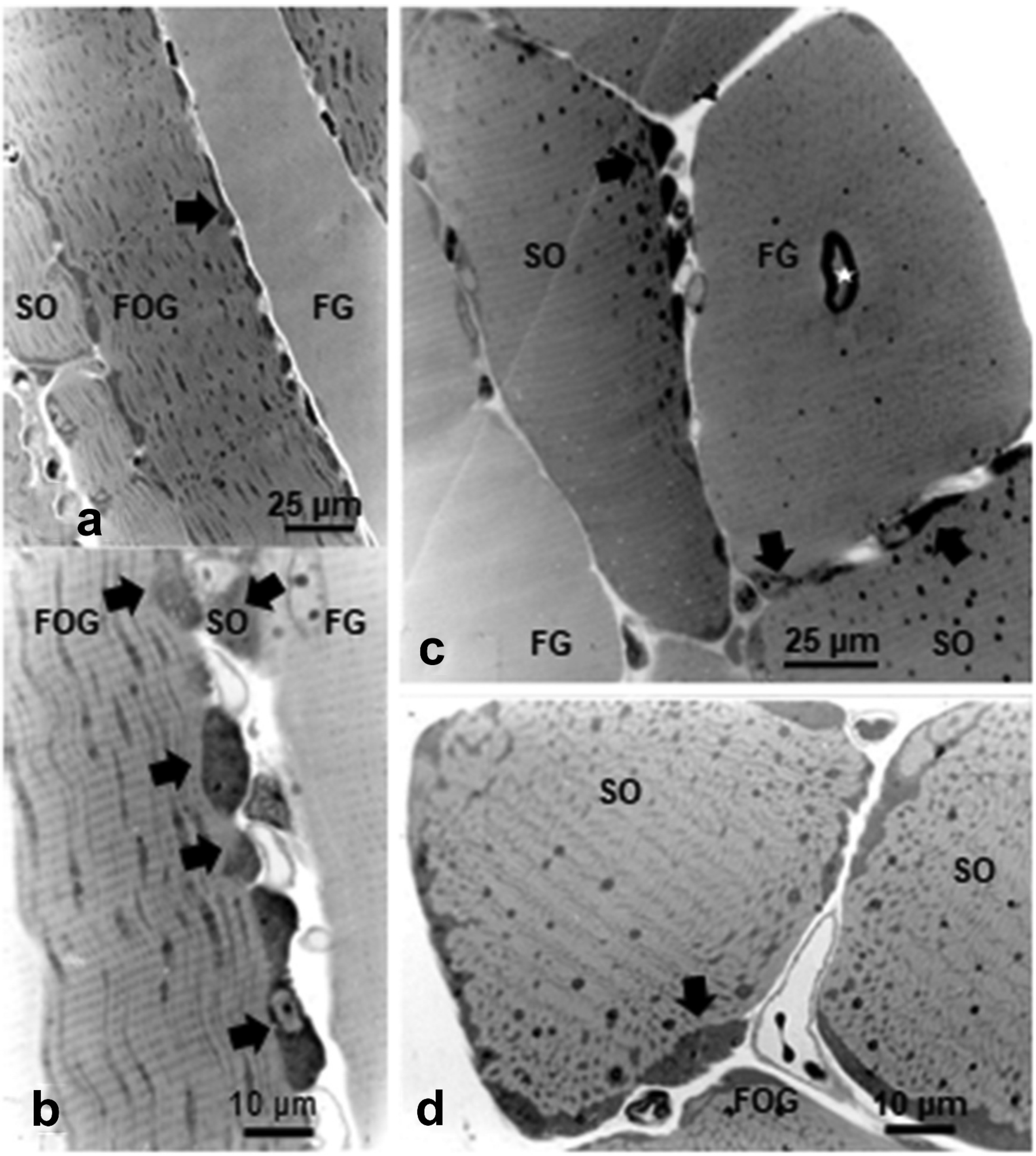

Figure 1. a-d: Pane of 1-µm thick longitudinal (a – c) and oblique cross-sections (b – d) of one 45 weeks old female obese Zucker tibialis anterior muscle, stained by toluidine blue. A cursory view reveals basophilic perikaryal and intermyofibrillar components in all the oxidative fibers, giving them a sort of 'ragged' aspect (black arrows). In overall, qualitatively, the muscle fiber diameters appeared as FG > FOG > SO types, whose diameter was verified quantitatively in (Figure 2(a-c)). b center displays a spot-fold artifact, not a central nucleus.

muscle fibers of the obese rats narrower than the lean muscle fiber of the same age female. Moreover, the histochemical markers and the histogram's comparisons between muscle fiber type measurement's distribution were illustrated (Figure 2(a,c)). There, the Kolmogorov-Smirnov tests, demonstrated with high significations that the quantitative measurements were normal

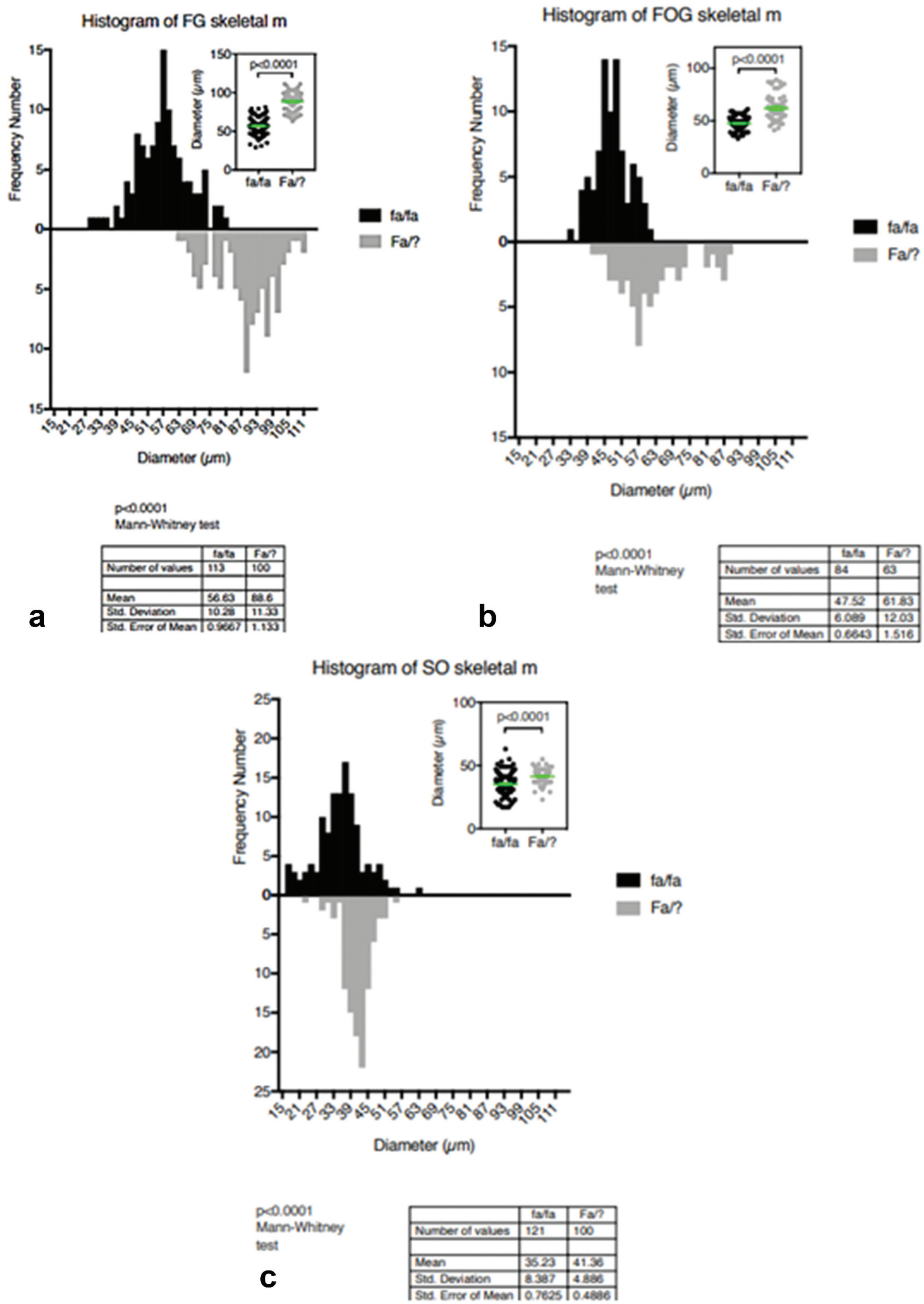

Figure 2. a-c. Comparative histograms of tibialis anterior muscle samples of Zucker obese and lean of the 45-week-old female rats and statistical comparisons indicate overall atrophy of the obese fiber types.

distributions ($p < .0001$) as well as the assumptions made with of histology qualitative aspects because SO or Type I fibers had 35.23 ± 8.387 µm in fa/fa (n = 121) vs 41.36 ± 4.886 µm (n = 100) with high significance (Figure 2(a)). The others, the fast oxidative glycolytic or intermediate type (FOG or type II A) in fa/fa measured 47.52 ± 6.089 µm (n = 84) vs Fa/? 61.83 ± 12.03 µm (n = 63) (Figure 2(b)) as well as the fast glycolytic type (FG or type IIB) revealed their narrow diameter in fa/fa 56.63 ± 10.28 µm (n = 113) to be still smaller than the ones of Fa/? having 88.60 ± 11.33 µm (n = 100) (Figure 2(c)). All the comparisons made between fiber types were verified with Mann-Whitney tests showing high significative meanings ($p < .0001$) Those comparisons between obese (fa/fa) and lean (Fa/?) fiber types of the tibialis muscles confirmed them to reveal and confirm the overall atrophy of the obese NIDDM muscles.

Transmission electron microscopy (TEM)

Out of LM 1-µm semi-thin sections (Figure 1(a)) of the muscle's samples, selected areas were used for ultrathin sections, as shown in the further figures. There, the 3 main skeletal muscle fiber types considered of the 45-week-old tibialis can be seen adjacent to one another, and even though already recognized with semi-thin sections, ultrastructure aspects made more comforting and new observations, especially about mitochondria and lipid deposits.

Subsarcolemmal and intermyofibrillar mitochondrial profiles

Accumulations of mitochondria profiles with adjacent osmiophilic deposits can be revealed in the outermost zones of the sarcoplasm and in the intermyofibrillar zones, also illustrated in all the Figures 4(a-c), 5(a,b), 6, 7, 8(a-d), 10(a,b), 11(c), 12(a) and 13(a). These accumulated mitochondria suggested and further confirmed that either the muscle profiles belonged to both oxidative fiber types, i. e. SO (type I) and FOG (type IIA) or fast fatigable or glycolytic as abbreviated FG, according to the histochemical profiles and fine aspects of this tibialis muscle fiber contents. The oxidative types have accumulations of the organelles but, especially, the FOG fibers would recall those described in muscle pathology as 'ragged.' However, if most of them do not seem to bear blemishes or altered microstructures at low magnification, the study of high magnification micrographs made us found peculiar scattered mitochondrial degradations with quasi obliteration or mitoptosis throughout the three fiber types (Figures 3, 4(a-c), 5(a,b), 6, 7, 8(a-d), 10(a,b), 11(c), 12(a) and 13(a) and Table 1).

The lipid depots (LDs)

The osmiophilic structures revealed different morphologies and can be subdivided into two types: (i) spherical fatty deposits (SDs) and (ii) interconnected subsarcolemmal or liposome-like bodies (Ls).

The spherical depots (SDs)

Specifically, large spherical fatty deposits ranging from 0.5 to 1.3 µm in diameter were usually located adjacent to mitochondria, either and both the subsarcolemmal zones or aligned with mitochondria in the intermyofibrillar sarcoplasm that belonged to both SO and FOG fibers (Figures 4(a-c), 5, 8(a,b), 10(c), 11(a)); however, these were only rarely viewed in the FG fibers (Table 2; Figures 3(a), 9(a) and 12(a, b), Table 1). In this subsarcolemmal location, some showed a bizarre and surprising eccentric degradation accompanied by debris (Figure 4(b)). With these accumulated mitochondria, SDs contributed the peripherally located festoons found in the LM sections (Figure 1(a-d)) that rendered the oxidative fibers as 'ragged.' It is only at the highest magnifications that these spherical structures appeared as lipid droplets without limiting membrane or apparent lining structure (Figures 5(a,b), 10(c,d), 11(a)). Their content displayed a sort of centripetal gradient of electron contrast reaching a lesser electron pale core as an amorphous blurry or mottled aspect, caused by the thickness of the sample's fixation, processing and sectioning (Figures 8, 10(c), 11(a), 12(a) and 13(c,d)). When detected in the subsarcolemmal zones, SDs showed a more uniform, full contrast but slightly distorted by the crowding with either the adjacent mitochondria profile and/or of a myofibril as well as the location of the triads (Figure 5(a,b)); SD distorted shapes revealed a homoeomorphic topology (Figures 4(a-c), 5(a,b),

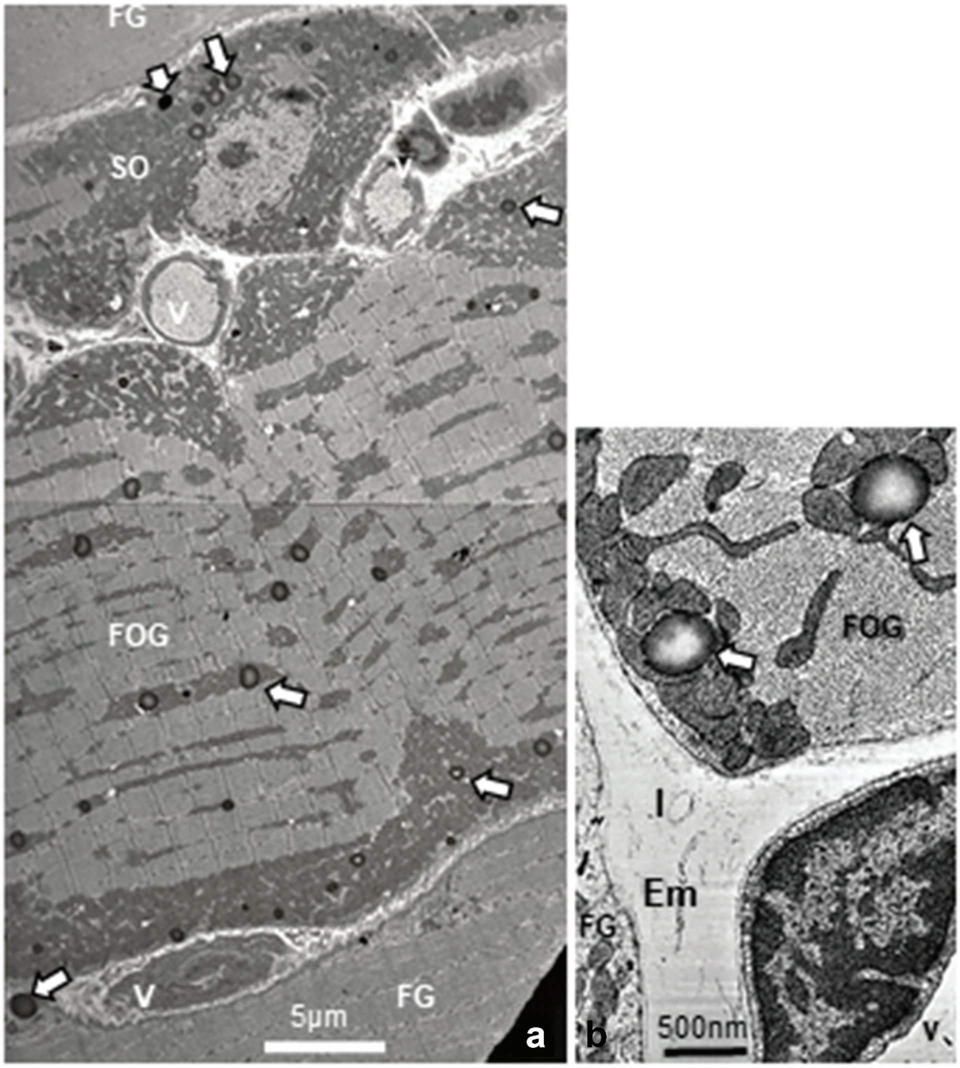

Figure 3. a-b: Pane of TEM aspects of 45-week-old obese female fa/fa tibialis anterior muscle showing parts of adjacent 3 main fiber types. Both SO and FOG fiber profiles typically contained mitochondria aggregates in the subsarcolemmal perikaryal and intermyofibrillar zones with large spherical lipid deposits (white arrows). FG fibers displayed only rare droplets (low left bottom arrow). b: Enlarged view of peripheral zone of an FOG fiber with spherical lipid depots (white arrows), adjacent to mitochondria. Em: Endomysium; FG: fast glycolytic fiber; v: blood vessel, l: lymphatic capillary.

Table 1. Comparisons between muscle tibialis sampled from obese (fa/fa) and lean (Fa/?) rats represented in Figure 2(a-c) as histograms.

Muscles	SO or Type I	FOG or Type IIA	FG or Type IIB			
n = 3	fa/fa	Fa/?	fa/fa	Fa/?	fa/fa	Fa/?
nb values	121	100	84	63	113	100
Mean Diameter	35.23	41.36	47.52	61.83	56.63	88.60
S.D.	8.387	4.886	6.089	12,03	10.28	11.33
s.e.m.	0.7625	0.4886	0.6643	1.5160	0.9667	1.1330

7, 9(a,b), 11(a), 12(a)). This deposit type is exceptionally found in the FG fibers (Figures 3(a),10(a), 12(a)). There, they were solitary and closely adjacent to the sarcolemma, often without a clear core.

The liposome networks (Ls) or interconnected subsarcolemmal deposits

Among oxidative fibers subsarcolemmal mitochondrial aggregates, other poorly recognizable deposits by LM aspects were only describable through fine structure aspects. Displayed as small lipid-like deposits, these Ls were highly and uniformly electron dense contrasted and their shapes varied; smaller than the SDs and found with the electron microscope as string-like accumulations recalling those of liposomes as lined by poorly-recognized lining membrane. Only found to the narrow perikaryal and sub sarcolemmal zones of the muscle fibers, their

Table 2. Obese female Zucker rat tibialis anterior muscle: mitochondria and spherical lipid deposits from 55–65 nm thick section's micrographs.

Skeletal muscle fiber types	SO fibers n = 3	FOG fibers n = 3	FG fibers n = 3
Surfaces of the illustrated fiber	40	2100	250
sections in μm^2	250	20	300
Total Section (μm2)	525	1250	90
	815	3370	740
Mitochondria			
Mitoptosis nb per total nb	10/123	5/86	6/25
mitochondria profiles in the	3/ 98	3/65	3/15
muscle fibers	15/269	15/365	8/15
Total nb mitochondria	490	516	55
Total nb mitoptoses	28	23	17
% Mitoptoses	5.71	4.45	30.90
Spherical Lipid Deposits (SDLS)			
Subsarcolemmal	1	1	1
SDLs nb	2	0	1
Total nb	1	3	1
Total Section's area (μm2)	4	4	3
% total Section	3.2005	3.1416	0.75
	1.5707	0.0932	0.0099
Intermyofibrillar	9	29	0
SDLs nb	12	4	0
Total nb	20	34	0
Total Section (μm2)	41	67	0
% total Sections	32.2014	52.6218	0
	3.9510	1.5614	0
Total section of SLDs (μm2)	35.4019	55.7634	0.75
% total Section	4.3438	1.6547	0.0993

n = number of muscle micrographs; nb: number; S: Surface section's measured.

fine structure features revealed them as if initiated near or by the small Golgi zones to form Ls (Figures 7, 10(a,b)). They also displayed interconnected oweverclumps of various shapes (Figures 6, 7, 9(a,b), 10(a)). With higher magnifications, Ls revealed a unit membrane lining with crenate aspect (Figures 6, 8(c,d)). In some oblique or tangential sections, the electron micrographs made up them of some aligned, interconnecting circular channels along their lining membranes, including those that contacted the outer membrane of the mitochondrial envelope (Figure 8(a-d)). In some cases, Ls were noted with a sort of hexagonal profile (Figure 8 (d)) among a filled to swollen homeomorphic endoplasm network (Figure 8(b)). Therefore, these Ls differed from the aforementioned SDs, those lacked lining membrane. In addition, the network of smooth endoplasm structures with electron contrasted content associated with the outer membrane's of the mitochondria envelopes through linkages or blunt conduits in continuity and their dense content of the intermembrane space, the inner membrane and the mitochondrial matrix (Figures 6 and 9(b)). Enlargements of some parts revealed in Figure 8(e,f) provide further details of the crenated lining of the Ls and its reticulum-containing complex that appeared to demonstrate elongated channel-like, resembling those found in vitro and in vivo, with lipids and phospholipids enriched by ceramides.

Mitochondria degradations and mitoptoses

Throughout all the muscles of the obese adult female Zucker rat, many of the mitochondria profiles showed either compacted matrices or with blurry aspects of matrices under high TEM magnifications in SO and FOG fibers (Figures 4(a-c), 7, 8(a,b), 9(a,b), 11(a-c)). Some others also showed scattered damage, and remnants of them. The damaged organelles were either swollen (Figure 4(a)) or both in part swollen and degraded (Figures 4(a), 5(a,b), 7, 8 (a), 9(a), 10(a), 11(a-c)) as well as entirely obliterated from the muscle fibers (Figures 11(a-c), 12(a-e)). Even with the small number of fibers illustrated throughout the illustrations collected of this report, we evaluated the ratios of mitochondria profiles degraded were most numerous in the FG fibers compared with both oxidative SO and FOG ones (Figures 12(a-d), 13(a-e); Table 1). In damaged organelles, inner membranes and cristae were still recognized within but in peculiar aspects, as illustrated by the pane of Figure 10(a-d), the mitochondrial remnants appeared as irregular morsels associated with electron dense droplets with a somewhat concentrically-aligned deposits as tiny electron dense deposits or granule-like with an accumulated centripetal-like pattern in the spaces made by the swollen or partially deteriorated mitochondria Internum or matrix (Figures 10(a-d), 11(a) and 14).

Endomysium

Most of the muscle fiber's basal laminae seemed to lack or were free from other typical

components of the basement membrane, i. e. collagen fibers of the endomysium, save

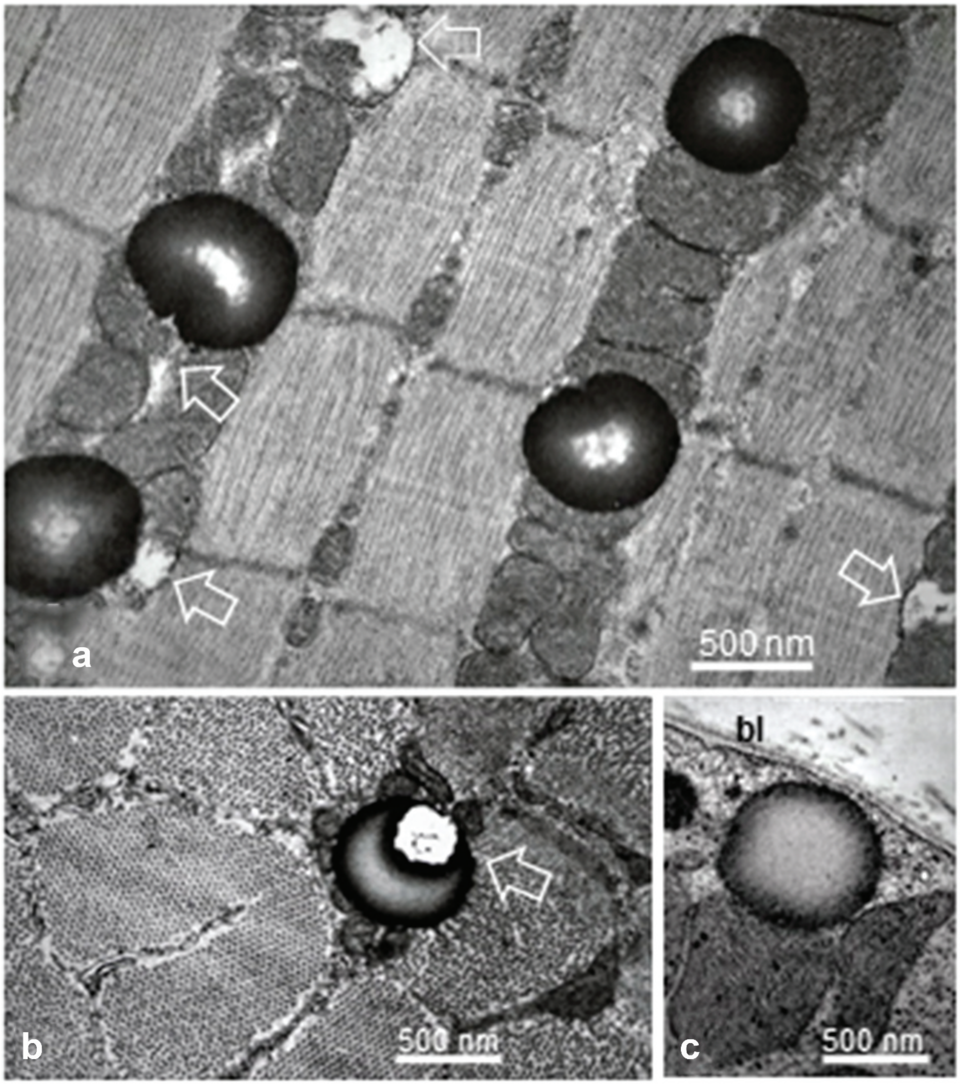

Figure 4. a-c: Enlarged spherical lipid deposits (SDs) located in intermyofibrillar location found in all SO or FOG fibers, among the rows of adjacent mitochondria as well as rare in subsarcolemmal position (c) and seldom found in FG fibers. White open arrows indicate damaged and degraded mitochondria throughout a and c. in b, a puzzling, eccentric fatty degradation between SD with mitochondrion. Note the centripetal gradient of oxido-reduced osmium contrast of all SDs. bl: basal lamina.

when muscle fibers were distant of less than 1-µm intercellular gap (Figures 5(a-c), 9(a,b), 10(a), 12(a)). Blood and rare lymphatic capillaries were often detected in the intercellular spaces.

Discussion

The *fa/fa* Zucker rats

Obvious somatic differences contrast the obese Zucker rats from the lean Zucker rat or any other lean 'strains' of laboratory rats, whether male or females due to their stooped posture and size, at rest, they bare their excessive weight. Furthermore, anesthetized, the huge adipose reserves made the rodents expand to appear as sorts of thick quiches.[9–12] Following the discovery of leptin, a product of secretion by the adipose cells, enterocytes and probably other unknown cells and tissues that influenced multiple CNS neurocrine centers and modulates other tissue's functions [20–33,54] including antidiabetic effects.[9–11] However, born without adequate leptin receptors,[12–14,17–20] these rats are driven by gluttony and undergo diabetes 2. Both male and female rats showed the same pattern of NIDDM ensued defects.[12–33]

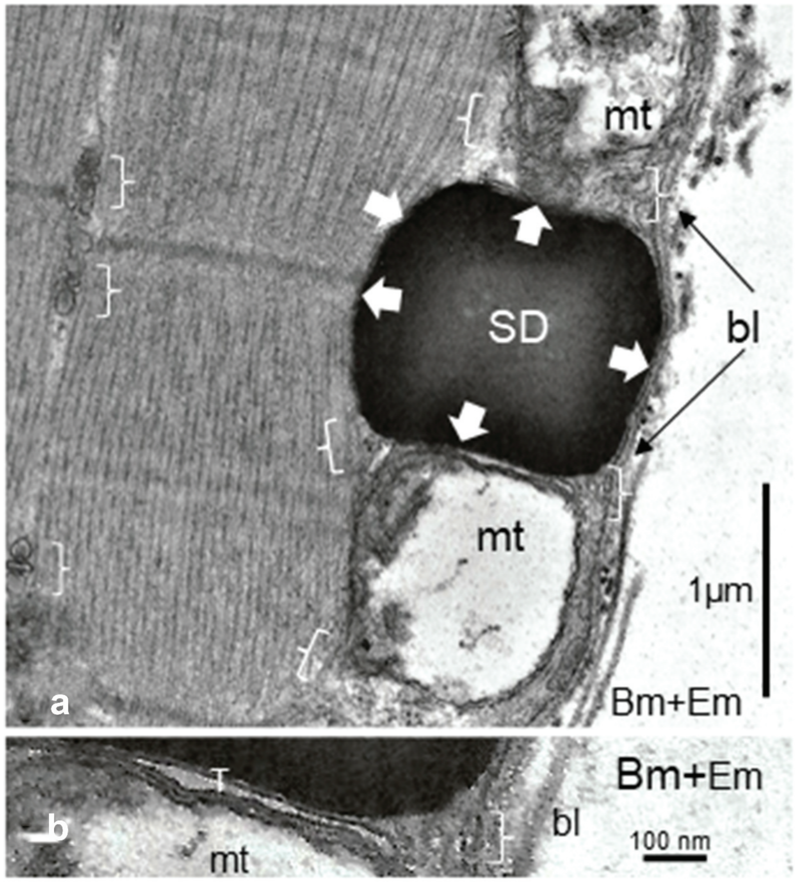

Figure 5. a-b Female Zucker FG tibialis muscle. a: SD deposit enlarged in the subsarcolemmal zone to view its inherent deformations (white arrows) caused by its adjacent muscle fiber substructures: sarcolemma, adjacents T-tubule (T), damaged mitochondria and myofibril. This later one also displayed a 'compressed' aspect, centered at and around its Z disc intersarcomere zone. Brackets: triad structures, including those displaced by deposit; bl: basal lamina; mt: mitochondrion. B: Enlarged aspect to verify the absence of membrane lining of the SD but T-tubule and part of mitochondria envelope are there, recognized. bl: basal lamina; Bm: Basement membrane; Em: endomysium.

Skeletal muscles and muscle fiber types

Skeletal muscle investigations have been achieved with animal models, invertebrates and vertebrates, including humans, through biopsies of patients and volunteered athletes. These abundant investigations allowed to comprehend both structure and functions of this bodily tissue, in deciphering its contractile fine machinery that the tissue has adapted with the skeletal frame for posture and locomotion e. g.[35,72–81] Out of these studies, using histochemical methods, at first, with toluidine blue alone[71,82] and, based on the intensity of staining at different pH levels, muscle fibers among muscles have been classified into 3 types, using myofibrillar ATPases and other mitochondrial dehydrogenases activities (such as succinic dehydrogenase (SDH)[49,68–72,74,75,83–89]; they provided a simple terminology as the SO (low ATPase, high SDH), FOG (high ATPase, high SDH) and FG fibers (high ATPase, low SDH). Ultimately, other studies subdivided human skeletal muscle tissue into seven human muscle fiber types, identified by myosin ATPase histochemical staining, from the slowest to the fastest ones: types I, IC, IIC, IIAC, IIA, IIAB, and IIB whose number's sequence corresponded from the 'red' or most 'oxidative' fibers (type I) to the most 'white' or 'glycolytic' ones as labeled IIB and, anatomically perceived from the most to least anatomical crimson tones corresponded to their relative content in myoglobin and mitochondria loads and activities.[49,80–89] Further refined biochemical techniques made ultimately 9 subtypes of the muscle fiber types to be recognized.[86–90] However, the adjacent subtypes tended to transiently convert into one another each other or to a main

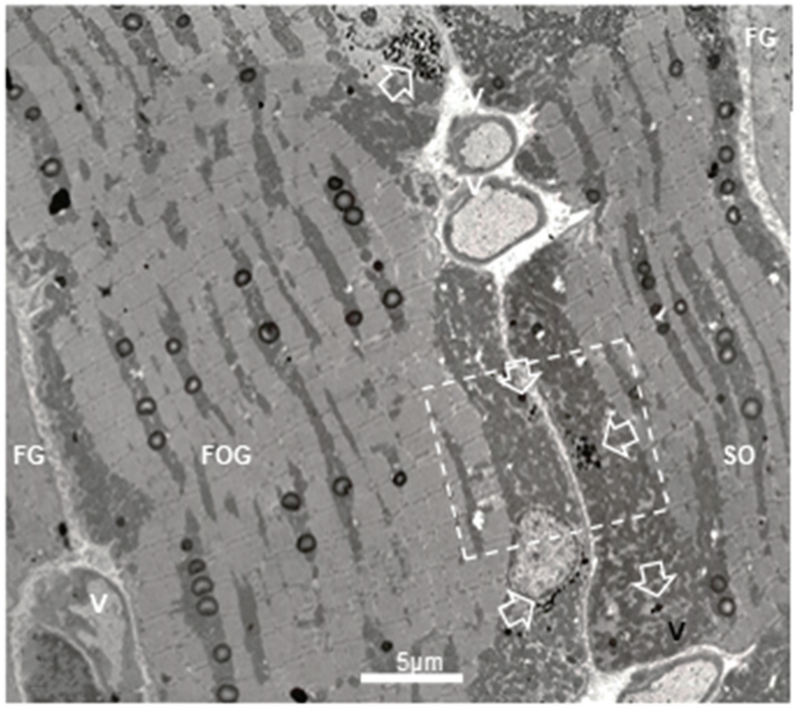

Figure 6. TEM montage pane of ibialis anterior muscle of obese female Zucker rat showing adjacent FG, FOG and SO fibers where a square indicated the field further enlarged in (Figure 7). White open arrows mark interconnected liposomes, only located in the subsarcolemmal and perikaryal muscle zones; rare SDs are also viewed. V: blood vessels.

functional 'type' according to the stimulated gene's expression(s) triggered after endurance and/or resistance training, with hypertrophy differences.[80,85,88] These can be used only for specialized study. However, these subtypes for any given muscle can be grouped differently by different researchers which created confusions between of published data comparisons. Thus, as cited and commented in several recent reports, most studies do not use these refined fiber types for making easier common ground of understanding between publications and categorize all muscle fibers into the 'original' three main fiber types.[68–70,83,84,89–95] Meanwhile, muscle genetic and histochemistry analyses demonstrated homologies between human and rodents e. g. [85–87, 89–94] and electron microscopy studies verified histology and fine structural homologies between rodents and human muscle fiber types [34,35,77–79,80,81,88–90, 92, 95–98]

The atrophy of the tibialis anterior muscle

As we followed the most common usage, we here reported about: (i) slow-twitch oxidative or type I (abbreviated SO), (ii) fast-twitch oxidative or type IIA (FOG) and (iii) fast-twich or type IIB (FG for 'fast glycolytic' or 'fast fatigable glycolytic') muscle fiber types. The rare, subtypes IIC, IIAC and IIAB were not even tried to be detected by special labelings, making less than 0.5% in this hindleg tibialis muscle. One has added to our initial reports about the obese Zucker rat muscles[57,58] and comforted other data on the same muscles where exercise physiology experiments were compared between male and female Zucker rats[99–102] and those about the same muscle without considering all 3 fiber types[93,103–110] by finding obese tibialis muscle fiber size demonstrated atrophy for all fiber types, 14.82% for SO fibers, 23,14% for FOG fibers and 24,79% for FG fibers respectively when compared to lean tibialis muscles whether in Zucker strain or other laboratory rats.[70,99,102,110–113] Overall, the obese Zucker muscle measurements found in this study supplemented other's data, such as those of poor incorporation of radiolabelled precursors,[112] reflected by decreased DNA and RNA contents,[113,114] likely hampered by deficient hypothalamo-hypophyseal signaling secretions caused by

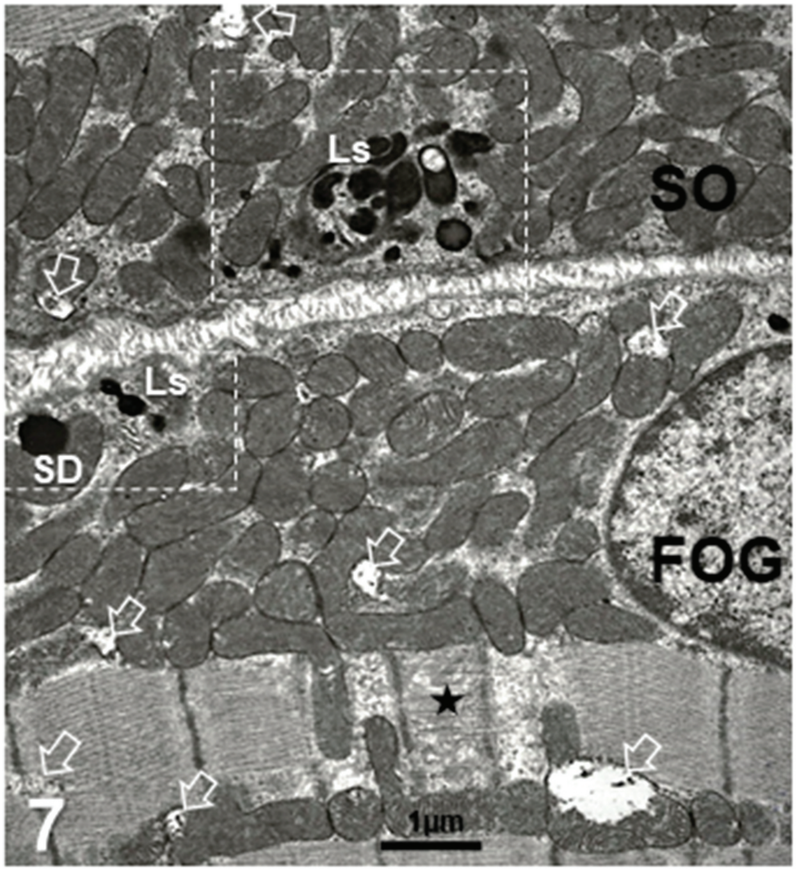

Figure 7. Ls and a single spherical deposit deposit in both SO and FOG fibers of an adult female obese Zucker rat (surrounded by broken rectangles whose enlarged views are displayed in (Figures 8(a) and 10(a-b)). Both fiber types contained mitochondria aggregates with a few degraded as marked with open arrows.

central leptin receptor defects, such as that of somatomedin (IGF-1), growth hormone (IGF-2)-[10,22–31,99,112–116] and still unknown factors, such some impeding bone[117] and insulin signaling and functions,[99–102,118,119] favoring thyroid gland changes[25,29–33,120] and poor vascular supply as parts of the defective muscle homeostasis ([108] vs [119,121,122]) and muscle atrophy that were also found in human diabetes 2.[123–125] This syndrome can be improved by exercise.[123] All the aforementioned data, including those of muscle, can also worsen with age in human cases[126,127] and the associated insulin resistance further increased by such sarcopenia.[125] The found muscle atrophy of the Zucker rat can be further comforted by a progression of tibialis nerve demyelination damages reported earlier, at younger age, where metabolites of sphingomyelin have been hypothesized, disrupting the myelin architecture [32,33]; see paragraph 4.b.

Skeletal muscle fine structure and diabetes obesity

Surprisingly, in human, only scarce but old studies have dealt with biopsies about fiber types[35–37,95–97,126,127] and after exercise[35,80,95,126,128] and too few about diabetes 2 [36,37] but included or based studies with only biochemistry aspects[7–9,125–129] even though utrastructural aspects would bring imagery resolution about crucial interpretative cell changes to verify and interpret some metabolic changes, like in many other tissues. A search through several specialized texts relevant to muscle defects confirmed this lack of human and animal ultrastructure data i. e. [130–135] Finding obesity-linked atrophy of the fiber types and serrated fringes of the oxidative fibers made us to further analyze fine features of the muscle samples with electron microscopy.

The distribution of both intramyofiber lipid depots LDs and SDs showed in our samples as well as those of young Zucker rats [99–102,112,113,136] corresponded to the known distribution in as

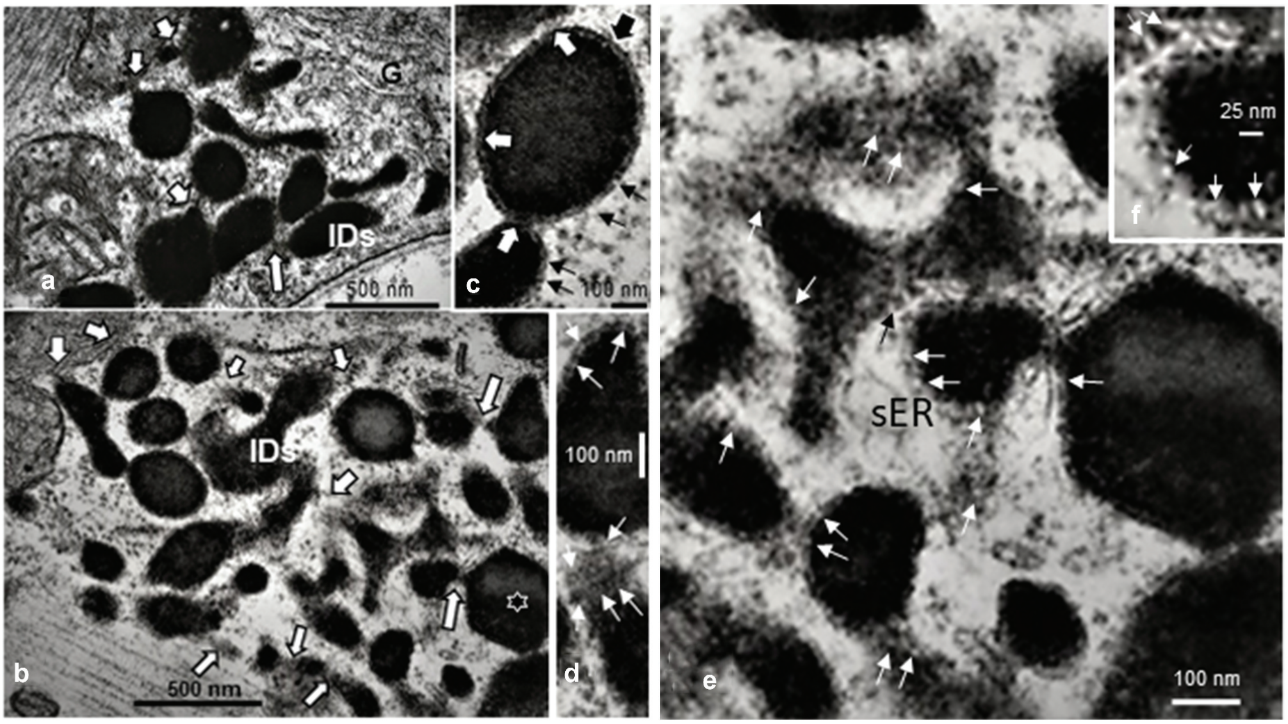

Figure 8. a-d: Example of liposome's aggregate (Ls) of 45-week-old female obese Zucker tibialis anterior muscle. a and b illustrate the numerous interconnecting bridges or channels appeared (white arrows) as a continuous reticulum contained the electron dense material that extended to the outer membrane of the mitochondria envelopes. In random sections, shapes of Ls varied in a sort of complex topology containing round to elongated ovoid into dodecahedron-like profiles (star) within the smooth endoplasmic reticulum. In c and d: Details of linings (in c, thick and white arrows) appeared and revealed a crenated aspect and, in d, further enlarged views of the same linings in oblique section formed sorts of circular, sieve-like aspect between encased pouch contents (thin white arrows) of similar size as those found ceramide-rich by others in vitro. g: Golgi; m: mitochondria. e-f: TEM enlargements of some parts of 8 b out of the previous pane revealed the crenated lining of the Ls complex (white arrows and a black on channel-like e). In f: micrograph further details demonstrated elongated channel-like, resembling those found in vitro, with phospholipids enriched by ceramides.

subsarcoplasm (SS) and intermyofibrillar (IM) lipid depots similar to those distributed in all typical mammal and humans[79–98,102,117,126–136] as well as those found in obesity and/or diabetes 2 cytopathology.[32,35,49,51,57,96,97,102,122,124–132] Other clinical studies have involved highly specialized imaging techniques, invasive or not invasive, and included the human tibialis anterior muscle as well as other muscles e. g. [112,113,126–128,136–142] with those of rodents, including the same Zucker rat model.[93,94,99–108,111–122,129,143,144] All confirmed the increased lipid depot distribution in diabetics and, surprisingly, the diabetic iris muscle was studied, even though, a smooth muscle.[145,146]

There, Zucker obese rat fatty deposit's distribution in fiber types was limited to red and white fibers[94,99,102,103,143] and other data showed there were no significative differences between human[34,36,45,49,51,80,96–102,134,136,143,144] with rodent's sex about muscle fiber type distribution.[35,93,103–107] One also can suggest that we found in old female diabetic rat's muscle ultrastructure could mirror unstudied old diabetic patients as a sort of incentive to pursue other human longitudinal studies. Additionally, leptin receptor models could also be created through gene knockout.

The lipid depots (LDs)

The LDs occurred in muscles like in many other tissues through coalescence from diffusion and endocytosis of extracellular heterogeneous hydrophobic dietary triglycerides, cholesterol metabolites and phospholipids sources.[35,49,126,129,147–150] These depots, as non-lined membrane droplets, in appearance unambiguous, with spherical profiles as those named here SDs, usually also accumulated small amounts of peculiar lipoproteins and proteins, including several that hedge these fatty droplets as perilipins. In the lipid-rich matter, some enzymes linked to signaling and lipid synthesis, RNAs along with lipid-soluble toxicants have been detected.[147] LDs have been described as 'inclusions' in cytology or as secretory 'milk' products in mammary glands for offspring.[148,149,151,152] These intracellular droplets can located adjacent to mitochondria profiles, like in muscles[35,36,77–81,84,87–89,91,96–98,126,128,150,153–156] and, in large quantities in the adipose tissues, specialized for lasting storages for energy source triggered through β-oxidative stimuli or other neuro-hormonal signals, yielding maximal output of ATPs in muscles for contractility[35–37,49,50,52,147,154] or, pathologically, with changed content, to alter the Krebs cycle output.[38–48,51,53,154–157] In all our fine structure data, using a similar cacodylate buffered fixative and processing of muscle samples, as done in previous studies where the lipid droplet's content appeared typically electron-lucent in muscle tissues as in other reports about other cells[148,158–161] or of young diabetic muscles[36] and, without using imidazole buffers as in,[158–161] the peculiar electron contrasted content with central mottled part appeared to strongly indicate high levels of ethylenic bounded components comprising unsaturated lipids or metabolites that enabled fixation to undergo oxido-reduction process of osmium tetroxide into osmates[162–166]; there electron contrast could be further increased by ceramide moieties involving high C numbers among the depots[153,154,165] as also found in diabetes with biochemical analyses[63] and commented in the next 4.b and 4.b paragraphs.

Classic LM examination of biopsies[83,84,88,91,130–135] along with magnetic resonance spectroscopy analyses showed an inverse relationship between accumulated lipids in human skeletal muscle tissues and insulin sensitivity for sedentary and obese humans where muscle LDs tend to increase.[60,136–142,155–157,167–177] The LD's distribution in the obese Zucker rat skeletal muscle have concurred with those found in humans[34,36,45,49,51,80,96–102,134,136,143,144,178] and the obese female Zucker rats, like in both sex of mammals and human diabetes models, have oxidative muscle fibers always containing significant more SDs comparably to those rare, SDs of the fast glycolytic ones with LM and fine structure[145] whose measurements are summarized in Table 1 and in Figure 13(a,b). These findings comforted this rat diabetes 2, along with other functional aspects documented with light microscopy, biochemistry and histochemistry.[13,14,36,45,60,80,96–98,102,134,136,143,144] The clarification of the so-called 'athlete's paradox' facilitated in the understanding as to how skeletal muscles utilize lipids and made authors to revisit the idea that lipid uptake with excess depots in obesity and diabetes 2 could contribute to insulin resistance[39,40,45,54,56,150,152–158,167–170,179,180] due to muscle's reduced and repressed oxidative enzyme's activities, respectively.[150,168,179,180] On the opposite, endurance training favored lipid uptakes and if LDs increased, sometimes more than in obesity,[60,153,154] these stores became efficiently used by an adapted, heightened, aerobic anabolism[156,171–178,181–189] caused by upregulated transcriptional activities, such as those of mRNAs of the hormone-sensitive lipase (LIPE), intramyocellular fatty acid's transport via muscle fatty acid binding protein (FABP3), and oxidative phosphorylation (cytochrome c oxidase I), including those of the tibialis anterior muscle,[59,60,150,174] all boosted through high-intensity interval aerobic exercises.[150,173–178,181–183,190] It was with quite similar findings in rats,[191] including the Zucker rats.[192–195] Phosphorylation changes of the coating surface proteins or lipoproteins such as perilipin 5, associated with oxidative fibers, including other organelles and the LDs can be modified by specific exercises that would adapt and improve the human NIDDM syndrome [152–155,181,196] while perilipin 2 is mainly with glycolytic fibers, in lesser amount and mainly located around the very rare LDs of FG fibers.[154,181]

SDs as Ls with ceramides?

LD's fine morphology of the found SDs suggested the admixed presence of other lipid-soluble electron-dense containing highly ethylenic groups with polar compounds in these old female diabetic muscles that could perturb the energetic capabilities of the organelles, impeding the normal utilization of lipids by the fiber's mitochondria along with or as 'insulin resistance' in dealing mainly with oxidative fibers. Among these, least metabolically active

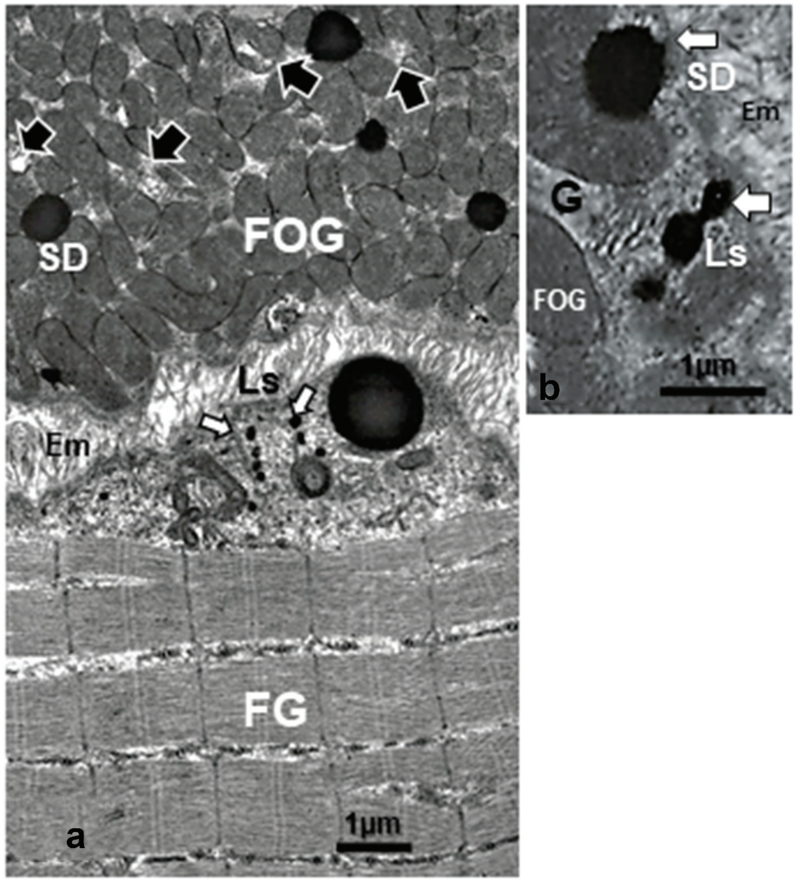

Figure 9. a-b: FOG and FG adjacent fibers of adult obese female Zucker rat tibialis anterior muscle. Note the FOG aggregate of mitochondria compared with the FG fiber (low part of 10A) devoid of such subsarcolemmal crowding but one SD showed closely adjacent to the sarcolemma. Both micrographs illustrate small aligned heavily contrasted strings of three vesicles in both fibers, marked by white arrows as Ls. A Golgi (g) zone could be involved with local endoplasm and process of capture and storage of these formed vesicles.

ones included long chain acyl-coenzyme As, diacylglycerol and ceramides.[39–46,59–63,191–195,198–202] Excessive lipid up taken caused by overfeeding and parts of the LDs, where ceramides originated from palmitate metabolism have lately received prominence after so much notices had focused on other lipids.[45,59–63,198–204] Ceramides in obese muscles could share multiple aspects in causing insulin resistance[62,205–207] through changing membrane surfaces,[208,209] displacing membrane rafts[206] with rearrangements of transmembrane channels[210] and changed other surface signalings[211] that could relate to the impeded mitochondrial respiration as 'resistance'[212] that can be reestablished by exercise, as discussed above.

An endoplasm reticulum filled excess amounts of inadequate metabolites, including lipids and ceramides as liposomes?

The sarcoplasm contains an endoplasmic reticulum highly specialized for fast ionic and energetic exchanger in muscles[35–37,77–81,83,84,97,98,130,131,213] and some part of it, like the sarcoplasmic reticulum, associated with ionic exchanges with myofibrils, could compartmentalize and specialize out of sarcolemma endocytosis and transcytosis, via Golgi apparatus and endosome-like, storage lipid sites. Obesity and hyperglycemia already make both increase in circulating shorter-chain saturated free fatty acids (FFA) that serve as substrates for and induce *de novo* ceramide synthesis[24,25,199–204] along with other complex lipids and cholesteryl esters captured by receptor-mediated endocytosis taken up from

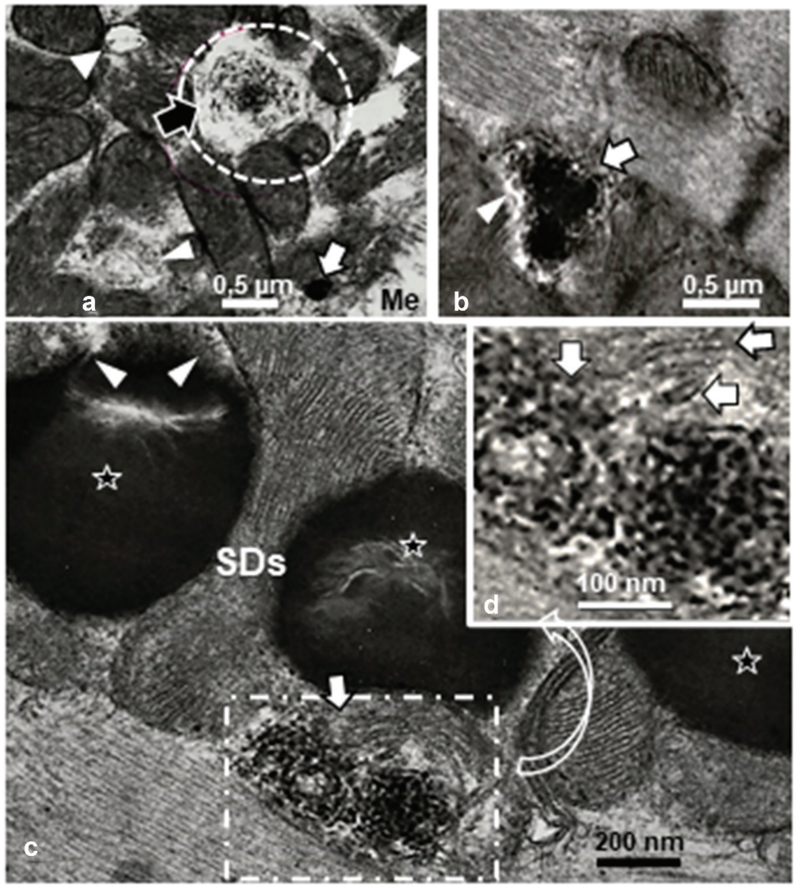

Figure 10. a – d: FOG and SO muscle fibers of adult obese female Zucker rat. Among the crowded intermyofibrillar mitochondria, degraded structures (arrowheads) bearing some concentric membrane whorls or stacks revealed what could be a filing by highly electron dense contrasted droplets ranging from 8–20 nm in diameter alongside those membranes (white arrows). d: Enlarged view of c demonstrates the centripetal-like trend of the aggregated deposits while becoming centrally coalescent and, thus, widened.

extracellular milieu (exiting circulation). Those can become parts of a reticulum of the endoplasm constructed with the subsarcolemmal Golgi apparatus (Figure 9(a,b)) into a membrane-enclosed lipids network of dynamic topology (as 'fixed' but illustrated in (Figures 6 and 8(e)). The string-like vesicles found in slow fibers resembled the chylomicrons found by others[214–216] – sometimes called liposomes – that resembled the same ones constructed artificially with double concentric amphiphilic lipid layers (phospholipids) that associate with water to form vesicles,[217,218] making 'nanoliposomes' to deliver medications,[217–220] including oligonucleotides (i.e., recent polyRNA vaccines against SARS-Cov 19). Ours are even more similar to those ceramides immunolabelled in keratinocytes.[221] Referring to our micrographs, accumulated liposomes enlarged by accretion and filled this swollen endoplasm network that acquire topologic variations of shapes, because of their corralled linings and as Ls, like in LDs, connected to the mitochondria oxidative 'furnaces.' Hence, those lipids enriched by complexed long-chain acyl groups, ceramides and their sphingosines 'escaping' autophagy through changes of perilipins[61–63,222] i. e. forming other electron- contrasted fine structures depots. Focusing about ceramides, the literature about them showed they not only overload the endoplasmic reticulum content but also its linings,[205–211,222–224] where membranes and intermembrane contacts in cross- and oblique sections showed peculiar crenated (<10 nm diam) to circular (10–25 nm in diam) formations in Figure 8 (c,e,f). These crenate aspects may relate to those reports that have not only detected membrane changes but also, in vitro verified channels made by accumulated ceramides including those found in mitochondria.[214,215,223,224] Whether or not ceramides or complex lipids, it is the first time, with

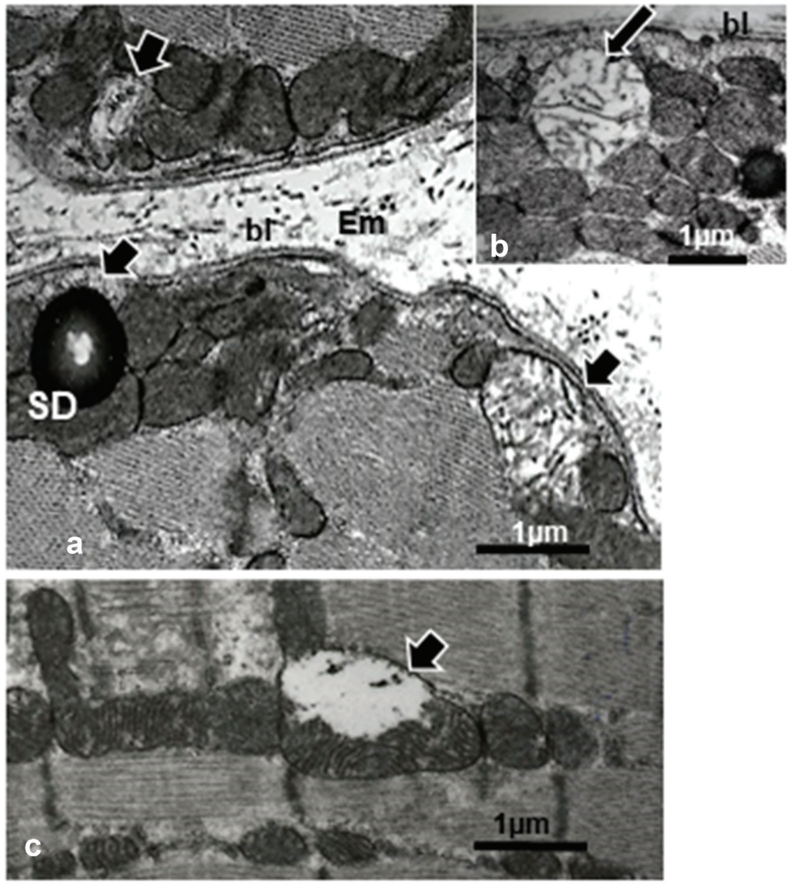

Figure 11. a-c: Typical mitolytic aspects or mitolysis in adult female obese Zucker rat tibialis anterior oxidative slow twitch (SO) and fast twitch or FOG muscle types. Profiles of sub-sarcolemmal (a-b) or intermyofibrillar (c) mitochondria revealed matrices either partially or entirely swollen-like degraded compared with other adjacent typical mitochondria profiles (white arrows). Highly contrasted SDs appear in both a and b. bl: basal lamina.

electron microscopy, that an 'endoplasm' displayed highly contrasted 'reticulum' or Ls network that reached and contacted the mitochondria outer membrane was detected in diabetes 2 muscles because LM aspects have not evidenced these structures yet.

Mitochondrial profiles and degradations as mitolyses and mitoptoses

As one noted in the above paragraph 4.c, the earliest reports dealing with human and animal model's investigations classically demonstrated the high content in mitochondria profiles along with lipid deposits in slow or oxidative fibers vs. those of glycolytic, fast twitch and fatigable. [35,36,77–81,64–83,97,98,130,131,225] However, token data collected about human and animal NIDDM/diabetes 2 muscle fine structure in specialized publications[35,36,126,128] could have been caused by LM poor resolution and marker's deficiency and, thus, may have reduced diabetes 2 interest with ultrastructure to befall focused on resolving metabolism. One also realized in our preliminary studies with LM alone that one disclosed atrophy and only glimpses of morphology alterations, similar to those that followed.[57,58] However, during the last three decades, molecular aspects have made so much strides and one would expect to provide interest for further longitudinal investigations in the TEM. Studies have clarified between the sub sarcolemma (SS) LDs and intermyofibrillar (IMF) LDs[153,154,220–222] along with specializations have been shown between them and the IMF and SS mitochondria due to proteomic and biochemical differences analyzed through mass spectrometry, because IMF LDs appeared to be the main fuel source for the IMF mitochondria that provide energy for adjacent myofibrils and sustained muscle contractility containing the highest levels of

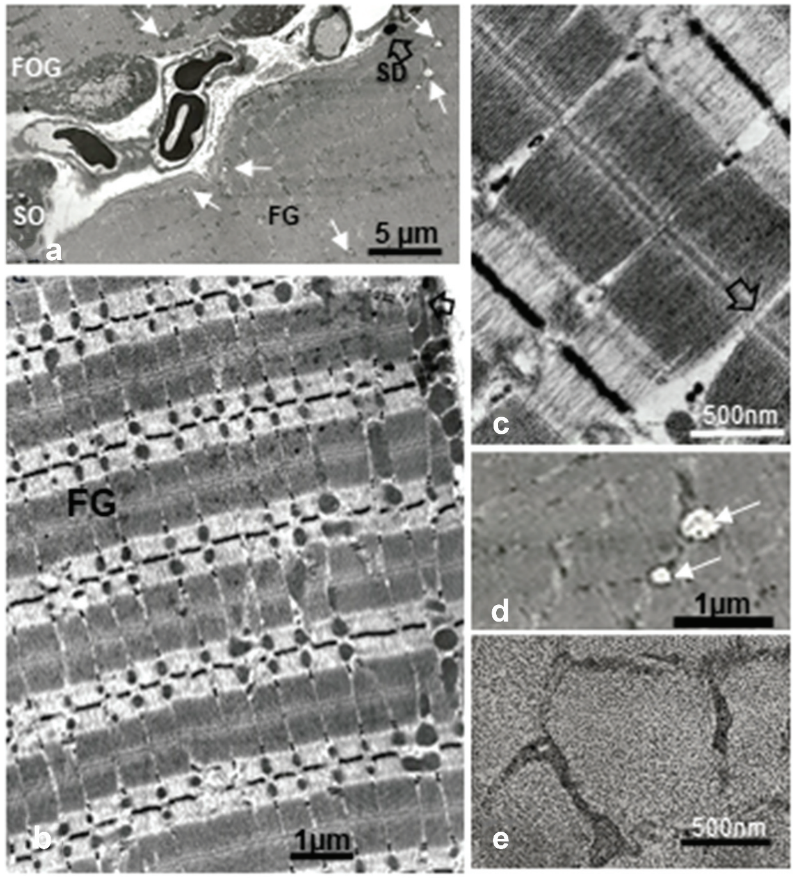

Figure 12. a-e: Obese female Zucker fast glycolytic (FG) muscle fiber ultrastructural aspects in cross, oblique and longitudinal sections. Typical myofibril architecture also showed throughout mitochondrial degradations as mitolyses or mitoptoses (small white arrows) and enlarged in d. These degradations occurred mostly within outermost regions of the myocytes. Notice throughout all the sections no glycogen aggregates (as in c, black arrows) showed in the intermyofibrillar sarcoplasm.

enzymes and phosphorylation proteins along with those respiratory chain complex while the SS LDs and mitochondria dealt with providing energetic demands for SS membrane related homeostatic and functions including those interactions-transports and dynamic exchanges of ions, metabolites of the adjacent endomysial space's.[14,60,66–81,153,220–224,226–228] Muscle fiber genome expression is also modulated by nerve influences, consistent with each fiber type and activity.[36,40,80,85,88,89,98,128,220–224,226–231] In the case of diabetes, palmitate metabolism yields ceramide and sphingosine compounds[59,61–63,198,199,211,229–231] and, probably, unlike of uninucleate cells, a pathway implicating reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the organelles implicated, those located in SS locations and some IMF ones induce cytochrome c escape that activates an 'apoptotic'-like pathway that swerve into mitochondrial fission and/or degradation known as 'mitoptosis' instead of mitophagy.[45–47,64,202,231,232] However, as seen in all muscle fiber types, the lytic degradations which cause(s) is (are) unclear – maybe peroxidations – [45,46,231–234] damaged matrices or internum and the inner membrane of the envelopes while most of the external membranes were left preserved, rendered resilient due to their remodeling with ceramides and/or metabolites, providing diverse type channels[235–240]. The linings and extensions of the Ls membranes could be loaded with the same ceramides or sphingosines as we revealed circular infrastructures, described in paragraph 4.c. These mitochondrial partially or entirely executed with cavitation of their matrix[47,148] can also become sinks of overloaded complex lipids as suggested in tethering them in the lucent remnants as in Figure 10(a-c) and measurements seemed to have indicated that

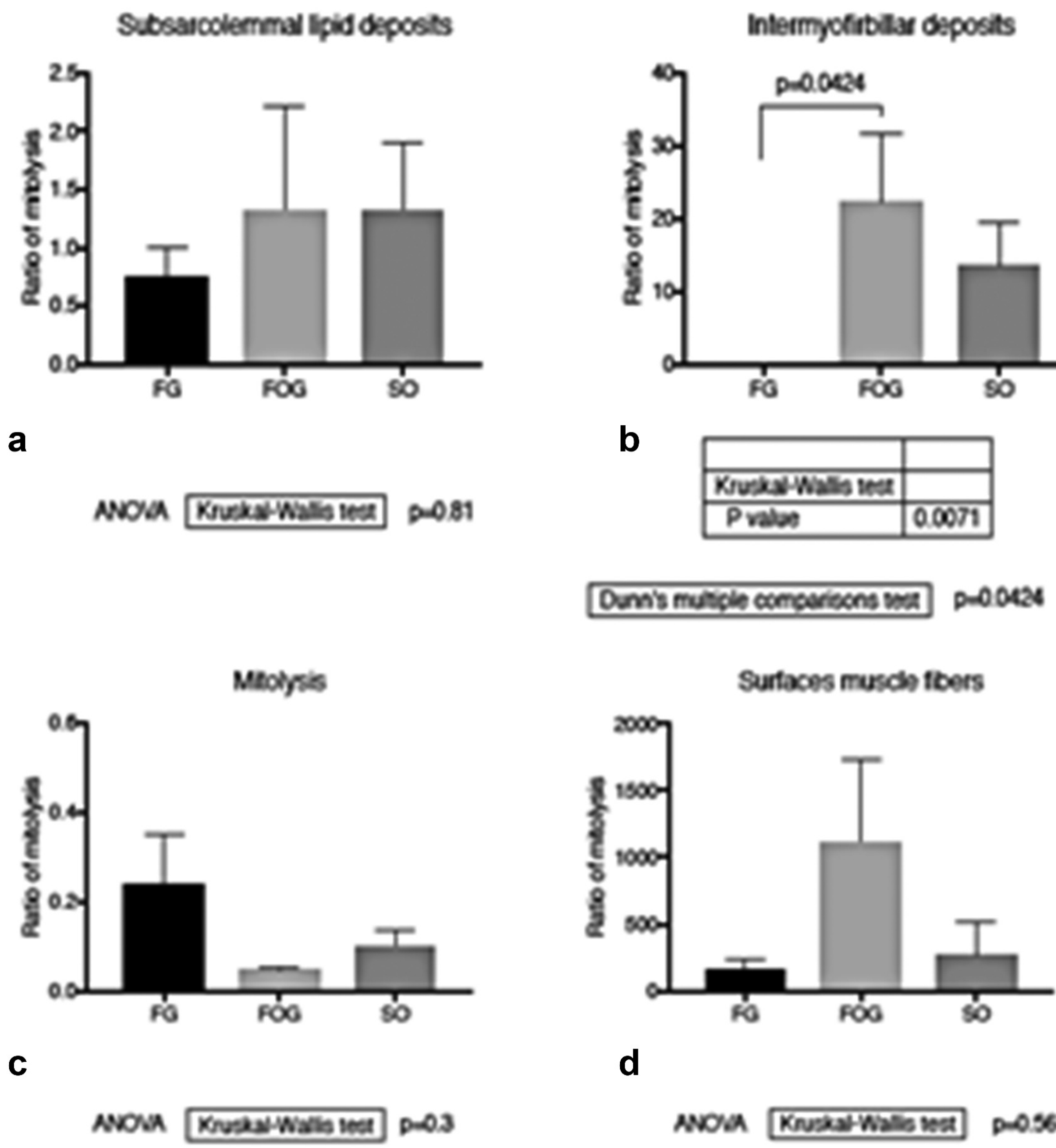

Figure 13. a-d: Comparative histograms from fine structure micrographic counts displaying more SDs in oxidative (SO and FOG) than FG fibers while none can be found FG fibers (a and b). In c: Mitolyses, including mitoptoses, are significantly more abundant in FG fibers than oxidative fibers. d: Surfaces of the 3 types of muscle ultrathin sections measured where a, b and c distributions were reported of the 45-week-old obese female Zucker rat tibialis anterior muscles.

for FG surfaces of sectioning measured, the number of mitolyses is significantly more important than in the oxidative fibers (Table 1 and Figure 13(c,d) where mitoptosis revealed only outer membrane of the envelope left. Could we hypothesize that, based on literature,[45–47,64,241] mitoptosis happened without apoptosis of the myocytes triggered by ceramides while myocytes were left to 'survive,' protected by their multinucleate structure that still control whatever can be in the diabetic syndrome mitochondria, with their altered their energetic capabilities.[199,200,212,213,225,228–234,237–243]

Diagrammatic representation in Figure 14 of the pane of (Figure 10(a-d)) suggesting the possibility of mitochondrion remnants as outer membrane, already natural 'sinks' for acylated lipids, gave in and yielded to become other lipid depots.

Conclusion and translational research considerations

Diabetes 2 as NIDDM syndrome condition of the female Zucker rat muscles showed to accumulate lipids and again demonstrated the validity of this

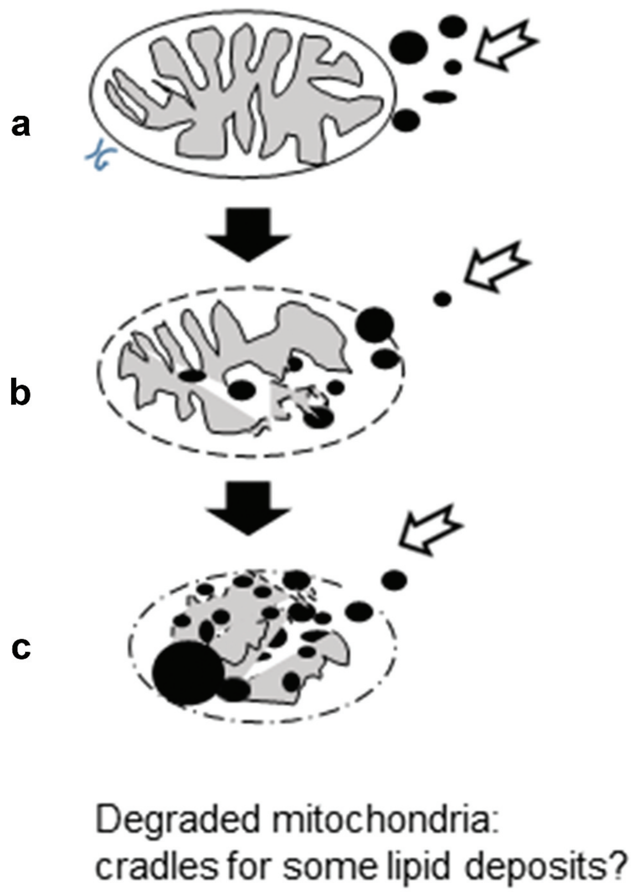

Figure 14. Diagrammatic representation suggesting the mitochondrion lytic remnant used as sink for diverse electron contrasted lipid and metabolites in oxidative muscle fibers.

rodent model to uncover some of the fine structure aspects associated correlated with peculiar metabolites depots[197] that could be involved in impeding sarcoplasm and mitochondrial anabolism, perturbing the insulin and other neurohormonal signals. Furthermore, our report concurred with the views of others[154,211] a that the regulatory mechanisms conferring lipid ceramide depositions[207] and utilization in skeletal muscle remain elementary and that more should be understood about musculature fine structure changes and gender along with aging in people afflicted by diabetes type 2, using interdisciplinary tools where fine morphology should be used along biochemistry markers for longitudinal investigations, including those of human biopsies, as it is done for other muscle diseases[244] Those could clarify functions and damages found as in Zucker rat model that may translate to further analyses of the human muscle's changes. These studies could bring into adjustments of the lipid-deficient or altered metabolism qualifying this public health syndrome and, could contemplate and assist as with the aging population with adjustment not only of seric glucose and carbohydrates nutrient's intake with new medications[10,245] but also, progressing with exercise and nutrients adaptations[37,51,246,247] along with further studies in rodents, whether with knockouts, and/or like with this rat model.[99,100,241,248,249]

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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