Adipose Tissue Remodeling by Prolonged Administration of High Dose of Vitamin D3 in Rats Treated to Prevent Sarcopenia

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To examine the effect of high dose vitamin D3 treatment on visceral adipose tissue, we used vitamin D deficient male Wistar rats (18 months old) as a model of sarcopenia. The aging process is not only responsive for the losing muscle mass but also for redistribution of lipid resulting in altered fatty acid storage and dysdifferentiation of mesenchymal precursors. The effect of aging and vitamin D treatment (weekly oral gavage with 0.125 mg vitamin D3 (5000 IU)/100g body weight) on the omental adipose tissue were histological examinated. At the end of the experiment (9 monhs), adaptive changes to the reduction of adipogenesis and increased apoptosis in response to long-term treatment with vitamin D consisted of smaller size of adipocyte and moderate macrophage infiltrate.

Key words: vitamin D, sarcopenia, visceral adipose tissue, intramuscular fat, remodeling

Fatty infiltration (intermuscular adipose tissue) and fibrosis are well-known histological feature of aging skeletal muscle and represent histopathological hallmarks of dystrophic muscle in humans [1-3]. Intermuscular adipose tissue is located between muscle groups and beneath the muscle fascia and intramuscular adipose tissue that is distributed within individual muscles. The fat within skeletal muscle includes most of the intramyocellular triglycerides, adipocytes present between muscle groups (intermuscular fat), and a smaller pool of adipocytes present between muscle fascicles (intramuscular adipocytes)[4-6].

Fat cells surrounding the muscle bundles could derive from different mesenchymal progenitors normally present in the adult skeletal muscle: mesenchymal stem cells, muscle-derived stem cells, or muscle satellite cells (SCs). Intramuscular accumulations of adipocytes of unknown origin also are observed in age-related sarcopenia, obesity, and type 2 diabetes and are associated with a decline in measures of muscle function [7]. In skeletal muscle, aging is manifested by gradual loss of muscle mass and function and skeletal muscle is eventually replaced by fatty and fibrous tissue. Sarcopenia is predicted to be the result of a loss of satellite cell number, or a failure of satellite cells to function in aged individuals [8]. Morever, in older age, redistribution of lipid to extra-adipose sites with aging could result from loss of lipid storage capacity in fat depots, altered fatty acid handling resulting in lipid accumulation, dysdifferentiation of mesenchymal precursors, such as muscle satellite cells is a potentially reversible process that could contribute to maldistribution of fat in old age [9, 10]. The potential of satellite cells to adopt different fates could be influence by the alterations in the muscle and systemic environment induced by the aging process [11].

The progressive infiltration of fat within the muscle tissue is associated with deterioration of muscle strength and functionality [11]. This fat infiltration may correspond to aberrant transdifferentiation of myogenic precursor cells into adipocytes resulting in the formation of fat within the intermuscular space.

Increasing evidence suggested a role of vitamin D supplementation on both myogenesis and adipogenesis. *In vitro* studies showed that the addition of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) to C2C12 skeletal muscle cells decreases cell proliferation and enhances myogenic differentiation through an increased expression and nuclear translocation of the VDR and modulation of pro-and anti-myogenic factors [12-14] In adipogenesis, the high concentrations of 1,25(OH)2D3 on 3T3-L1 preadipocytes resulted in lipid accumulation and inhibition of expression levels of adipogenic specific genes [15, 16]. In animal studies, the effect of excessive vitamin D supplementation could reduced accumulation of body fat mass [17]. By contrast, vitamin D deficiency contribute to skeletal muscle atrophy [18] and increased adiposity [16, 19].

Adipogenic transdifferentiation of muscle satellite cells by addition of increased doses of 1,25(OH)2D3 was also examined [20]. Low physiological concentrations of 1,25(OH)2D3 increased fat droplet accumulation, whereas high physiological and supraphysiological concentrations inhibited fat accumulation.

Little is known on the effect of prolonged treatment with high physiological doses of vitamin D on muscle regeneration and adipogenic transdifferentiation of satelite cells *in vivo*. Aging rats have many characteristics in common with humans regarding progressive changes in skeletal muscle architecture and selective loss of type II

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alpha motor neurons and fast fibers with age [21-23]. Aging rats start losing muscle mass in their lower extremities at ~18 months [24]. The animal models of sarcopenia used in this report are vitamin D deficient aged rats (more than 18 months old).

The purpose of this study was to examine the pathohistological changes of omental adipose tissue in vitamin D deficient animals and the effect of high dose vitamin D3 treatment.

Experimental part

Animals

Twenty young (N=10, 3 months old, 128±13g) and old (N=10, 9 months old, 290±24 g) male Wistar rats were purchased from the Cantacuzino Institute, Bucharest, Romania. Rats were randomly distributed in four experimental groups with five animals per group: 1. young vitamin D deficient, YD(-); 2. old vitamin D deficient, OD(-); 3. young vitamin D treated, YD(+); 4. Old vitamin D treated, OD(+). The experiment respected the instructions of the general guidelines for the care and use of laboratory animals, recommended by the Council of European Communities [25]. All experimental procedures were approved by the Laboratory Animal Care Committee of the Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania.

Protocol of vitamin D deficiency and vitamin D treatment

The pre-experiment acclimatization of the rats was assured by hosting them in constant temperature (22°±1°C) and humidity (60%) conditions, and the circadian cycle (12h light/12h darkness). The animals were kept for 7 days in the laboratory conditions and were daily monitored and clinical examined for possible disease conditions or abnormal behavior.

After the acclimatizatin period, the young and adult rats were housed in polypropylene cages and maintained on a 12 h light/12 h dark cycle under ultraviolet B-free incandescent light to minimize endogenous vitamin D production. All rats were allowed free access to laboratory food pellets (Cantacuzino Institute, Bucharest, Romania) and fresh clean drinking water at all time. All four diets had similar carbohydrate, total fiber, protein, and fat contents (table 1).

Table 1

STANDARD DIET (g/kg)

Fat	50
Crude protein	180
Arginine	4.3
Asparagine	4
Glutamic acid	40
Histidine	2.8
Isoleucine	6.2
Leucine	10.7
Lysine	9.2
Methionine+cystine	9.8
Phenilalanine+threosine	10.2
Threonine	6.2
Tryptofan	2
Valine	7.4
Calcium	5
Cloride	0.5
Sodium	0.5
Phosphate	2
Vitamin A	1.2 mg/kg
Vitamin D3	0.025 mg/kg
Vitamin E	27 mg/kg
Choline	750 mg/kg

The young and adult animals were randomly divided in control and treated vitamin D groups. The animals were kept under similar conditions except that the vitamin D groups received weekly via oral gavage vitamin D (Vigantol oil, KGaA, Darmstadt, Germany, 0.5 mg vitamin D3/mL), in dose of 0.125 mg vitamin D3 (5000 IU)/100g body weight and vitamin D deficient groups received palm oil [26].

The body weights were monitored weekly. The duration of the experiment was 9 months.

Sarcopenia model

Knowing that the presence of age-related changes is more pronounced in late life, beyond the 18 month age [24], we conducted the experiment in Wistar rats for a period of 9 months, at the end of the experiment the adult animals reached 18 months (old rats). The combination of vitamin D deficiency and age-related dmuscle dysfunctions was for the first time used as a model of sarcopenia.

Animal necropsy and processing of samples

At the end of the experiment, animals from all groups were euthanized with isoflurane and the gross examination has been performed. The surgical procedures on animals were performed in the Surgery Department of Faculty of Veterinary Medicine, Iasi, by a specialized surgery team.

The omental fat was immediately removed, weighed and rinsed with ice-cold phosphate buffer saline (*p*H 7.2). Subsequently, the samples had been processed at the Molecular Biology laboratory of the Regional Oncology Institute of Iasi, Pathophysiology Laboratory and Pathology Department of the Faculty of Veterinary Medicine Iasi.

Histological analysis and morphometry

All samples were fixed in 10% buffered formalin and embedded in parafine with a tissue processor Leica TP1020 (Leica Microsystems GmbH, Germany). Sections of 5μm thickness were obtained with a Microtome SLEE CUT 6062 (SLEE Medical GmbH, Germany), deparaffinized and stained by the bichrome (Hematoxylin-eosin) techniques. The qualitative histology was performed from stained sections using a light microscope Leica DM 750 (Leica Microsystems GmbH, Germany) with an attached digital camera Leica ICC50 HD (Leica Microsystems GmbH, Germany) Germany). The photos were taken with Leica Application Suit Software (LAS) version 4.2.

In this study, the pathological aspects of adipose tissue were assessed: adipocyte shape (aspect), infiltrate of macrophages in the adipose tissue with a crown-like structure and vascularization of the adipose tissue and the area surrounded by macrophages.

Morphometric parameters (area and major axis) were determined in tissue sections by computer-assisted image analysis software [27, 28]. For each sample, 100 adipocytes from different sections were analyzed.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Student's t test and Bonferroni's multiple comparison test (Statistical Software Package SPSS®, version 13, SPSS Inc., Chicago, IL, USA). Unpaired Student's t-tests were performed to determine whether there were significant differences between groups ($p<0.05$).

Results and discussions

Measurement of body weight

The table 2 shows the evolution of the weight in the studied groups. Rats in the vitamin D deficient groups had

Table 2

MEAN ± SD VALUES FOR WEIGHT IN STUDIED GROUPS

Group		Week 0	Weeks 1-36								
			S1	S3	S6	S12	S24	S36	Δ1	Δ2	Δ3
YD(-) n=5	$\bar{x}$	141	220	260	348	408	438	440	217	26,7	7,4
	SD	8	25	39	51	63	85	85	61	4,5	3,1
	Min	130	180	198	266	310	318	320	129	20,3	3,2
	Max	150	240	292	398	476	512	510	270	30,3	10,4
OD(-) n=5	$\bar{x}$	295	333	346	377	444	464	465	57,7	23,4	4,5
	SD	10	13	10	12	26	26	26	9,9	4,1	1,4
	Min	286	318	336	362	402	422	422	45,5	16,6	2,2
	Max	310	346	362	392	470	494	492	69,7	26,9	5,8
YD(+) n=5	$\bar{x}$	115	197	231	299	392	450	458	309	51,8	15,4
	SD	23	19	23	19	28	32	31	86	12,0	5,1
	Min	82	160	190	278	344	400	404	189	37,4	9,2
	Max	160	230	262	332	428	510	518	449	82,4	24,5
OD(+) n=5	$\bar{x}$	284	310	328	377	430	439	436	60,2	19,0	1,3
	SD	28	29	27	30	20	31	31	11,8	5,2	4,3
	Min	260	264	288	340	386	400	394	19,7	11,3	-6,2
	Max	382	398	412	462	458	468	462	71,4	26,0	6,0
FANOVA test (p)		0.001	0.001	0.001	0.001	0.001	0.006	0.589	0.001	0.001	0.001

Δ1 – the percent change S36 months vs S0; Δ2 – the percent change S36 months vs S12; Δ3 – the percent change S36 months vs S24
P values:Two way ANOVA, $p<0.05$
Groups: young vitamin D deficient, YD(-), old vitamin D deficient, YD(-),young vitamin D c, YD(+), old vitamin D treated, YD(+).

body weights higher than the vitamin D treated group all over the experiment period (fig. 1).

Histological examination and adipocyte size of omental adipose tissue

The changes in histological aspects and adipocytes size are illustrated in figures 1-4.

Muscle fat infiltration can be the result of aberrant transdifferentiation of myogenic precursor cells into adipocytes, resulting in the formation of fat within the intermuscular space. Recent studies demonstrated that myogenic precursor cells have the potential to transdifferentiate towards the adipogenic lineage [5] and

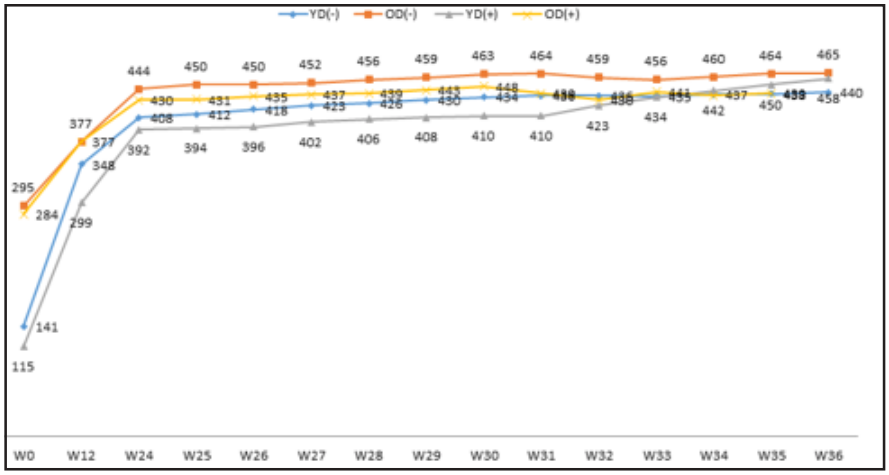

Fig. 1 . Evolution of the mean values of weight in animal groups during the experinental period.
Groups: young vitamin D deficient, YD(-), old vitamin D deficient, YD(-), young vitamin D treated, YD(+), old vitamin D treated, OD(+).

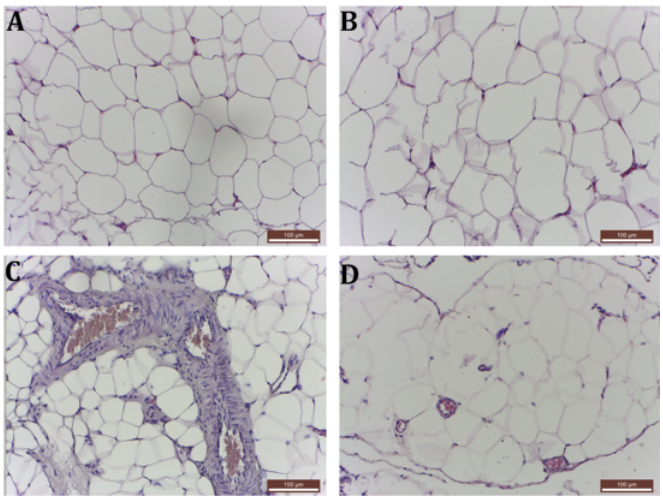

Fig. 2. Light microscope images of hematoxilin-eosin stained omental adipose tissue at 200X magnification. A. Young vitamin D deficient, YD(-), B. old vitamin D deficient, OD(-), C. young vitamin D treated, YD(+), D. Old vitamin D treated, OD(+). Vitamin D deficient rats (A and B) show large, clear and round-shaped adipocytes with tensed cell membrane due to excess triglycerides; interstitial capillaries are slightly compressed by the adipocytes. No mesenchymal cells are present. Vitamin D treated rats (C and D) show rare, smaller, polygonal-shaped adipocytes with slightly rough cell membranes; the ectasia of interstitial blood vessels and their overload with red blood cells; moderate cellular interstitial infiltrate containing fibroblasts, lymphocytes and macrophages.

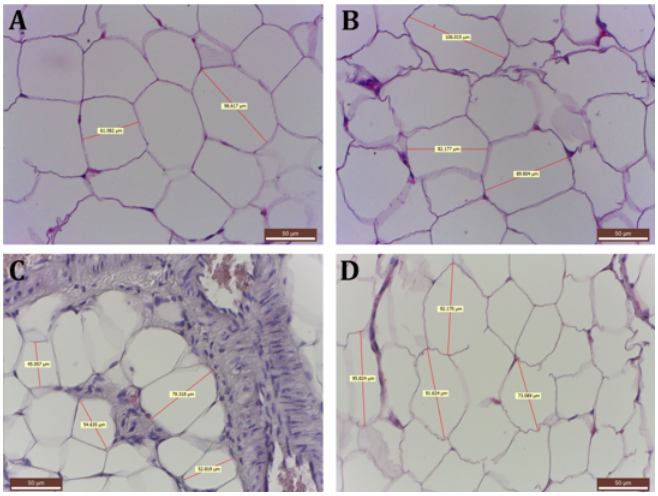

Fig. 3. Major axis measured on the cross-sectional areas of hematoxilin-eosin stained omental adipose tissue at 400X magnification. A. Young vitamin D deficient, YD(-), B. old vitamin D deficient, OD(-), C. young vitamin D supplemented, YD(+), D. Old vitamin D supplemented, OD(+).

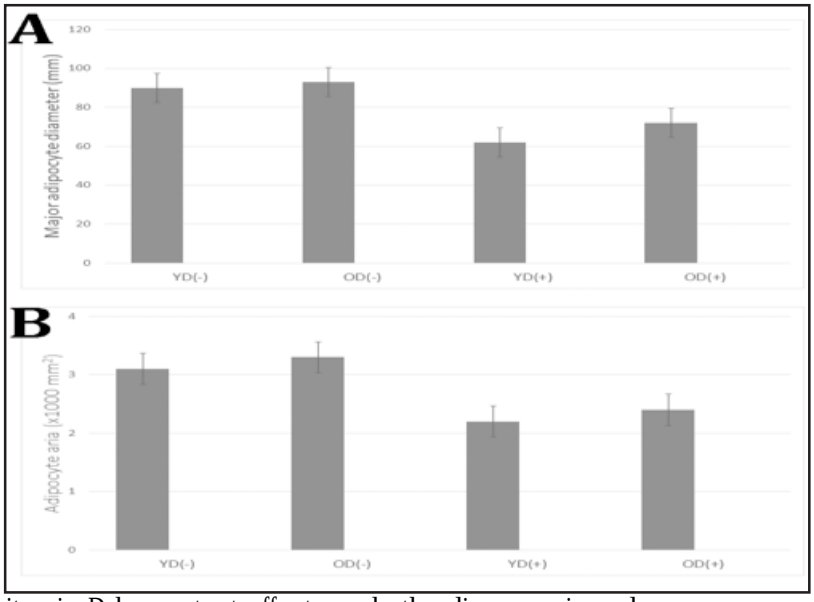

Fig 4. Adipocyte size, major diameter (A) and aria (B), was analyzed with an image analysis system and quantified. All values are the mean ± SEM (N = 5 rats/group).

vitamin D has potent effects on both adipogenesis and myogenesis [12]. Muscle fat infiltration has been shown to have a direct consequence on muscle strength and functionality, but it is also a key independent risk factor for metabolic diseases, such as insulin resistance, and sarcopenia [20, 29]. Our study used vitamin D deficiency and aging-induced muscle disfunction in old male Wistar rats as an experimental model of sarcopenia .

Vitamin D, a fat-soluble hormone, is able to regulate the transcription of many genes through vitamin D receptor (VDR)[29]. Muscle and adipose tissues are recognized as a target for vitamin D actions. In adipose tissue, adipocytes may be directly involved in the local synthesis and degradation of calcitriol, which is able to affect adipocyte biology by modulating pre-adipocyte differentiation, lipid accumulation and mobilization and also adipokine production. There are contradictory data in the literature regarding the role of 1,25(OH)2D3 in adipogenesis [30] but there are studies indicate that liganded nVDR is involved in the inhibition of adipogenesis [31]. 1,25(OH)2D3 treatment blocked adipogenesis in VDR+/+ cells but failed to do so in VDR-/-cells [31].

In this study, we used 0.125 mg vitamin D3 (5000 IU)/ 100g body weight which was administrated by oral gavage one time per week during nine months. The dose was in the upper physiological range since the rodenticide dose is considered 2 mg/100g body weight/day [32]. The prolonged high dose vitamin D3 treatment had an inhibitory effect on adiposity, the animals receiving vitamin D treatment had lower weights as compared to vitamin D deficient rats (fig. 1).

The mean value of major diameter of adipocytes in the omental adipose tissue of vitamin D treated rats was less than in vitamin D deficient rats (fig. 4), thus reflecting reduced fat mass. The aria of adipocytes in the vitamin D treated groups decreased, being proportional to the decrease adiposity and defective lipid storage. Moreover, at the end of the experiment, the young and old animals treated with vitamin D showed smaller, polygonal-shaped adipocytes with slightly rough cell membranes; the ectasia of interstitial blood vessels and moderate inflammatory interstitial infiltrate.

Our data suggest that the adipose tissue undergoes dynamic remodeling consisting in quantitative alterations in adipose tissue-resident cells. Changes in the number and size of the adipocytes could affect the micro-environment of fat tissues. Moreover, stromal vascular cells in the adipose tissue, including immune cells, are involved in numerous adaptive processes, such as dead adipocyte clearance, adipogenesis, and angiogenesis [33].

The remodeling of adipose tissue produced by prolonged high dose vitamin D treatment suggest that there is a dose and temporal effect of vitamin D addition to adipogenesis [31]. The reduced adiposity and aria of adipocytes produced by vitamin D treatment could be the result of inhibition of adipogenesis and induction of apoptosis [30, 34]. The additional studies demonstrated that 1,25(OH)2D3 increased fat mobilization by reducing intracellular fat contents and increasing lipolysis [15].

In the muscle tissue, the long-term high dose vitamin D3 used in this study was associated with decreased

intramuscular fat and stromal steatosis of the muscles compared to vitamin D deficient old animals (unpublished data). The cummulated histological aspects observed in adipose and muscle tissues in vitamin D treated rats suggest that loss of lipid storage capacity noticed in the omental fat was not associated with ectopic deposition of fat (i.e. intramuscular fat).

Conclusions

Vitamin D plays an important role in signaling in both muscle and adipose tissues. The prolonged addition of vitamin D to prevent the loss of function in muscular tissue could produced dynamic remodeling of adipose tissue. Adaptive changes to the reduction of adipogenesis and increased apoptosis in response to long-term treatment with vitamin D consisted of smaller size of adipocyte and moderate macrophage infiltrate.

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