Muscular dystrophy-related quantitative and chemical changes in adenohypophysis GH-cells in golden retrievers


Abstract

Duchenne muscular dystrophy (DMD) is a recessive X-linked lethal condition which affects a boy in every 3300 births. It is caused by the absence of dystrophin, a protein occurring especially within the musculoskeletal system and in neurons in specific regions of the central nervous system (CNS). Growth hormone (GH) inhibition is believed to decrease the severity of DMD and could perhaps be used in its treatment. However, the underlying pathological mechanism is not known. The golden retriever muscular dystrophy dog (GRMD) represents an animal model in the study of DMD. In this paper we investigated the morphological aspects of the adenohypophysis as well as the total number and size of GH-granulated cells using design-based stereological methods in a limited number of dystrophic and healthy golden retrievers. GH-cells were larger (32.4%) in dystrophic dogs than in healthy animals (p = 0.01) and they occupied a larger portion (62.5%) of the adenohypophysis volume (p = 0.01) without changes in either adenohypophysis volume (p = 0.893) or total number of GH-granulated cells (p = 0.869). With regard to ultrastructure, granulated cells possessed double-layer electron-dense granules which were evenly distributed in the cytosol. Furthermore, these granules in dystrophic animals occupied a larger proportion of GH-granulated cell volume (66.9%; p = 0.008) as well as of all GH-cells in the whole pars distalis of adenohypophysis (77.3%; p = 0.035), albeit IGF-1 serum concentration was lower in severe cases. This suggests difficulties in the GH secretion that might possibly be associated to dystrophin absence. In contrast to earlier reports, our data suggest that a lower IGF-1 concentration may be more related to a severe, as opposed to a benign, clinical form of muscular dystrophy.

Keywords: Stereology; Duchenne muscular dystrophy; Golden retriever; Hypophysis; GH

1. Introduction

Golden retriever muscular dystrophy (GRMD) is a degenerative myopathy which affects a specific breed of dogs and is genetically homologous to human Duchenne muscular dystrophy (DMD) [1]. Both dystrophies are inherited as X-linked recessive traits and characterized...
by the lack of dystrophin, due to a frame-shift mutation in the DMD gene. Dystrophin is a cytoskeletal protein of smooth, cardiac and skeletal muscles that anchor cytoskeletal actin to extracellular laminin [2,3]. Homologues of DMD have been identified in several animals including dogs, cats, mice, fish and invertebrates [3]. The most notable of these are the extensively studied mdx mouse, a genetic and biochemical model of the human disease, and the muscular dystrophic GR dog, which is the nearest pathological relative of DMD [4–6].

The growth hormone (GH) is secreted by somatotrophic cells of the adenohypophysis, and is essential to regulation of the corporal metabolism, including the muscular system. It has been postulated that patients with a benign clinical form of the disease may have a deficiency of GH [7–10] and then GH inhibition may deter the progression of the dystrophic process resulting in a milder clinical form of the disease [7–10].

The absence of the cytoskeletal protein dystrophin may lead to failure in the exocytosis process which causes instability of the plasma membrane. This feature provokes morphological alterations in the organelles and in the vesicles’ dynamics, apart from changes in the maintenance of the intracellular calcium [1–3,5,8,11–15].

The primary aim of this study was to investigate changes in the GH-granulated adenohypophysis’ cells (somatotrophos) in GRMD, such as their total number, numerical density and size by using design-based stereological methods. These data were correlated with circulating IGF-1 levels in an attempt to better understand the pathogenesis of muscular dystrophy.

2. Materials and methods

2.1. Animals

A total of eleven male golden retriever (GR) dogs, four healthy (non-dystrophic) aged from 7 to 12 months and seven dystrophic (GRMD) aged from 7 to 12 months were investigated in the present study.

It has to be stressed that animals were divided into two groups: healthy and dystrophic strictly based on three simultaneous and complementary criteria: genotyping analysis, creatine kinase (CPK) levels and clinical diagnosis which were therefore based on the existence or absence of clinical symptoms. The first two criteria are the most important since clinical symptoms occur only during the first month of life, and at birth both genotyping analysis [17] and CPK can reveal the presence of GRMD [3,17–20]. Therefore, GR dogs were considered healthy only in the absence of muscular dystrophy based on all criteria stated above.

According to clinical symptoms, dystrophic animals were further classified in two categories: severe clinical form (five animals) and benign clinical form (two animals). The clinical symptoms used to classify dystrophic animals were the severity of: dyspnea, dysphagia, muscular weakness and diarrhea.

2.2. Hypophyses

For the histological study two healthy adult dogs and three severe clinical form dystrophic adult dogs were used. All subjects were obtained from the kennel of the Department of Surgery of College of Veterinary Medicine at University of São Paulo (USP), Brazil, in association with the Genome Centre of Institute of Biosciences at University of São Paulo (USP) for the study of the muscular dystrophy in Golden Retriever dogs (GRMD).

One non-dystrophic dog died as a result of complications caused by a femoral fracture and the other, as a consequence of femoral dysplasia. These two events were, therefore, not linked to GRMD. Other dystrophic dogs died as a result of GRMD, e.g. respiratory dysfunctions. Non-dystrophic animals died at the age of one year and dystrophic animals died at 10–12 months of age. It should be stressed that these five animals were not euthanized. For IGF-1 serum concentration measurement, four dystrophic GR dogs and two healthy GR dogs were used.

The histological (stereological) study was pursued in those five adult animals quoted above and not in those used for IGF-1 study due to the fact that we did not have access to donated adult animals before our kennel was built and IGF-1 dosages should start at birth and be followed up to the fourth or seventh month of age. However, we had the results of creatine kinase (CK) and PCR Genotyping (see below) from these adult dogs donated by Genome Centre of Institute of Biosciences at University of São Paulo (USP).

Hence, we waited until the first generation of dogs to initiate the IGF study which came after the stereological study.

That is the reason why we used two sets of animals, i.e. one for the stereological investigation and another for the IGF-1 study. It would have been better if we had access to all animals once to see whether or not there would be a correlation between the stereological data and the data obtained from the IGF-1 study.

The whole experiment was approved by the College of Veterinary Medicine’s Animal Care Committee (application number 480/2004). It should be stressed that the total number of animals used either for the stereological study (five) and those used for the IGF-1 study (six) were restricted by our Animal Care Committee which prohibited us to euthanise the animals used for the IGF-1 study leading to a smaller number of animals available for this purpose.
3. Methods

3.1. Creatine kinase (CK) serum concentration

GR dogs serum samples were obtained from all animals (4 non-dystrophic and 7 dystrophic) by veno puncture at five different time points, i.e., = at birth, in the first, second, third and fourth week after birth, and then collected in tubes, centrifuged and aliquoted for determining creatine kinase CK concentration. The determination of CK was achieved by applying a method which consists in evaluating the CK catalysis using a CK enzymatic assay kit (Sigma Diagnostics, St. Louis, MO, USA).

The reaction is conducted through a CK catalysis which transfers a phosphate group from creatine phosphate to adenosine diphosphate. The resultant adenosine triphosphate is then measured by using two associated reactions, catalysis by hexokinase and glucose-6-phosphate dehydrogenase, which produce nicotinamide adenine dinucleotide (NADH) [21, 22].

3.2. GRMD PCR genotyping

Genomic DNA was extracted from blood samples of newborn Golden Retrievers (from the same animals tested for CK) with a commercially available kit. The genotypes of wild-type, carrier, and affected dogs were determined according to Sharp et al. [23] and Honeyman et al. [16] using GF2 and GR1 primers. The GF2 primer (5'-CTT AAG GAA TGA TGG GCA TGG G-3') corresponded to base pairs 135–114 of the canine dystrophin gene (intron 6) and the GR1 primer (5'-TGC ATG TTC CAG TCG TTG TGT GGC-3') corresponded to base pairs 805–782 (exon 7). The PCR product containing the Sau 96I site created by the mutation in the dystrophin gene was digested, electrophoresed, and visualized with ethidium bromide.

To characterize the defect in GRMD, a single-stranded dystrophin cDNA was amplified after being prepared from skeletal muscle of normal and affected dogs. Specific oligonucleotides were used in separate reactions to prime the reverse transcription of canine total RNA. All primers for the PCR reaction were based on the human dystrophin cDNA sequence and proved suitable for the amplification of all segments of the canine dystrophin mRNA. After the resulting cDNA had been purified, it was subjected to amplification by the polymerase chain reaction (PCR) [24] using the original reverse oligonucleotide primer paired with one of the forward oligonucleotide primers (F1 or F2). Products amplified by PCR were visualized on an ethidium bromide stained agarose gel and subsequent hybridization with human skeletal muscle dystrophin cDNA [25].

3.3. IGF-1 serum concentration

IGF-1 levels from six GR dogs: four GRMD (two having a benign and two possessing a severe clinical form) and two healthy animals were analyzed. All GR dogs came from the same family background. Distinctions between benign and severe clinical form GRMD dogs were mainly based on clinical symptoms and examinations (see above statements).

From newborn GR dogs (healthy and dystrophic), blood was monthly sampled during the first, second, third, fourth, and the seventh month of age. Nutritional conditions were kept similar for all animals due to the fact that it may interfere in IGF-1 serum concentration [26]. IGF-1 was determined by RIA after extraction with ethanol using a kit for human IGF-1 dosage (Diagnostic Systems Laboratories, Webster, TX). Human and dog IGF-1 present a high homology (95.2%) which allowed us to use a human IGF-1 kit to determine dogs' IGF-1 serum concentrations. No cross reactions for IGF-2, insulin, pro-insulin and GH [27, 28] were seen. All tests were performed in duplicate in the same assay and the intra-assay variability was 3.2%.

3.4. Histology

After death, animals were perfusion-fixed by 4% formaldehyde in PBS solution (pH 7.4; 0.1 M). Later on, hypophyses were dissected out by a trans-sphenoidal access and measured for length, width and thickness using a digital pachymeter Digimess.

The neuro-hypophysis was isolated from the adenohypophysis. By means of random sampling, right or left hemi-adenohypophyses (pars distalis) were used for immunohistochemistry and treated by the following standard procedure (see Section 3.6) and the other hemi-adenohypophyses (used for stereology) were kept in a modified Karnovsky solution (5% glutaraldehyde + 1% formaldehyde) in sodium cacodylate buffer (0.125 M) (pH 7.4) for 72 h, dehydrated in crescent series of ethanol and embedded in Araldite 502 Resin (EMS) followed by exhaustive and serially sectioning at every 50th section (K = 50) on a RMC ultramicrotome (TM RL) into 2 μm thick semi-thin sections. Sections were collected on glass slides, dried on a hot plate (Leica), stained with a 1% alcoholic toluidine blue solution and mounted under coverslips.

For the transmission electron microscopy the same protocol described above was followed, though ultrathin sections were obtained at 70–90 nm and collected afterwards in 200 mesh-grids. The sections were stained with 3% uranyl acetate solution in ethanol and lead citrate in order to be observed in a JEOL Electron Microscope JEM-1010.
3.5. Stereology

The stereological aim was to obtain estimates of GH-granulated cells (somatotrophs) numerical density, total number and volume as well as the volume of their granules. Systematic uniform random (SUR) sampling was used to generate tissue sections.

3.5.1. Numerical density of GH-granulated cells, \( N_{V} \)

The disector method was used to estimate the numerical density of granulated cells in a hemi-adenohypophysis (pars distalis) was given by multiplying \( N \) in a whole adenohypophysis (pars distalis) was given simply multiplying \( N \) from right or left adenohypophysis, which was chosen systematically and randomly, by 2.

The error variance of number estimation was estimated as shown in [31,32]. The error variance of number estimation was 0.05 for healthy dogs and 0.04 for dystrophic dogs.

3.5.2. Adenohypophysis volume, \( V_{Hypo} \)

The fractional volume of adenohypophysis occupied by GH-granulated cells was determined by point counting. A SUR sample of fields was selected and test points were randomly superimposed. We counted the total number of points falling within the adenohypophysis \( \sum P \) (ref) and the total falling on the GH-granulated cells \( \sum PGH cells \). Volume density was then estimated simply as:

\[
V_v = \frac{\sum P(\text{GH - cell})}{\sum P(\text{Hypo})}
\]

The error variance for ratios \( V_v \) was estimated according to [32].

3.5.3. Total number of GH-granulated cells, \( N(\text{GH-cell}) \)

The total number of granulated cells was estimated by multiplying \( N_v \) by the volume of the hemi-adenohypophysis \( V \): \( N = N_v \cdot V \). The total number of the cells in a whole adenohypophysis (pars distalis) was given simply multiplying \( N \) from right or left adenohypophysis.

3.5.4. Volume density of GH-granulated cells, \( V_v(\text{GH-cell/Hypo}) \)

The fractional volume of adenohypophysis' GH-cell occupied by its granules was determined by point counting. At EM level a SUR sample of fields was selected and test points were randomly superimposed. We counted the total number of points falling within adenohypophysis' GH-cells \( \sum P \) (ref) and the total falling on their granules \( \sum PGH cells \). Volume density was then estimated simply as:

\[
V_v = \frac{\sum P(\text{GH granules})}{\sum P(\text{Hypo})}
\]

The error variance for ratios \( V_v \) was estimated according to [32].

3.5.5. Mean GH-granulated cell volume, \( \bar{V}_N(\text{GH-cell}) \)

This was estimated from the ratio between the volume density \( V_v \) and the numerical density \( N_v \) of GH-granulated cells which, since the reference volume (adenohypophysis volume) is common, is equivalent to dividing the total volume of granulated cells by their total number.

3.5.6. Volume density of GH-cells' granules, \( V_v(\text{GH granules/Hypo}) \)

The fractional volume of an adenohypophysis' GH-cell occupied by its granules was determined by point counting. At EM level a SUR sample of fields was selected and test points were randomly superimposed. We counted the total number of points falling within adenohypophysis' GH-cells \( \sum P \) (ref) and the total falling on their granules \( \sum PGH cell \). Volume density was then estimated simply as:

\[
V_v = \frac{\sum P(\text{GH granules})}{\sum P(\text{Hypo})}
\]

The error variance for ratios \( V_v \) was estimated according to [32].

3.5.7. Total granule volume per GH-granulated cell, \( V(\text{granules, G cell}) \)

The total volume of granules within adenohypophysis' GH-cells was estimated indirectly by multiplying GH-cells' granule volume density by the mean GH-granulated cell volume \( v_N(\text{GH-cell}) \).
3.5.8. Total granule volume in all adenohypophysis’ GH-cells, \( V(\text{granules}) \)

The total volume of granules within adenohypophysis’ GH-cells (pars distalis) was estimated indirectly by multiplying the total granule volume per GH-granulated cell \( (V_G) \) by the total number of GH-granulated cells \( (N) \) and by 2 due to systematic and random choice by left or right adenohypophysis.

3.6. Immunohistochemistry

Hemi-adenohypophyses (left or right, by random choice) were frozen in Tissue Tek (Sigma) followed by isopentane (Sigma) and liquid nitrogen and cryosectioned in a cryostat Cryocut (Leica). Glands were equally cryosectioned (2 µm-thickness) keeping the same interval \( (K = 50) \) used for the contralateral hemi-adenohypophysis used for stereology (see Section 3.4).

Every 50th section was sampled together with its two neighbours (51st and 52nd) generating a trio of sections. In this study design, the first section was immunologically labelled to identify GH-cells, the second section was stained with toluidine blue to figure out cell profile morphology and whether these cells would contain granules and the third section was used as a control of the immunoreaction (omitting the primary antibody).

In addition, the first and second sections were compared to toluidine blue stained granulated cells (symmetrically localised) in the contralateral hemi-adenohypophysis which was only chosen for stereology. The correspondence between the latter and those GH-immunolabelled and toluidine blue stained cells (in the contralateral hemi-adenohypophysis) was based on two criteria taken altogether, i.e., (i) the immunolabelling of GH-cells, (ii) symmetric position and comparison of cell profiles between GH-immunolabelled and toluidine blue stained granulated cells (in one hemi-gland) and toluidine blue stained granulated cells (chosen for stereology) in the contralateral gland.

By adopting this procedure, we could assure that the granular cells considered for morphometry and stereology were, actually, GH-cells.

Immunofluorescence reaction was carried out by blocking sections with normal horse serum for one hour, incubate overnight in primary antibody, i.e. rabbit anti-rat GH diluted in PBS (1:50), (Amersham), washed in PBS solution (pH 7.4; 0.1 M), incubated in biotinylated secondary antibody donkey anti-rabbit in PBS (1:250) (Amersham) for 2 h, washed in PBS and finally, incubated in streptavidin–fluorescein in PBS (1:100) (Amersham) for one hour and washed in PBS. Slides were mounted with citifluor AF1 (Citifluor) and analyzed in a DMR Leica microscope coupled with a Leica® DFC 300F digital camera and Leica Q-Win Image Analysis Software (Leica Microsystems Imaging Solution Ltd., Cambridge, UK). As stated above control reactions were performed simply omitting the primary antibody and following the same steps afterwards.

3.7. Statistical analysis

The statistical analysis was conducted according to the nature of the parameters to be investigated. For continuous distribution data (cell volume and granule volume) and cell counting (discrete distribution data), i.e. total number of cells, the analysis accounted for the comparative effect between groups (dystrophic versus healthy dogs), using one-way ANOVA and General Linear Models \( (F\text{-test}) \), through a special procedure of the Minitab, version 14 [33] and Statistical Analysis System software (SAS), version 8.02 [34]. Normal distribution was assured by means of two procedures, i.e., (i) Shapiro–Wilk and Anderson–Darling tests and (ii) normal plot graph of residuals carried out by means of Minitab, version 14 and SAS [33,34]. In case of \( p < 0.05 \), \( T\)-test and Tukey tests were applied for multiple comparisons.

For IGF-I serum concentration, the statistical model considered two principal factors: age (1–7 months) and group (dystrophic versus healthy). This analysis was conducted by means of two-way ANOVA test and \( T\)-test for multiple comparisons \( (p < 0.05) \). The choice for a parametric test was made given the same reasons pointed out above, though the sample size was also small.

4. Results

Results are shown as mean (CV), where CV equals SD/mean.

4.1. GRMD genotyping

Genotyping analysis of GRMD dogs, i.e. dystrophic subjects showed that a mutation in the dystrophin gene produced a novel Sau 96I recognition site and the digestion of the 310 bp genomic PCR was used to diagnose the mutant allele. The wild-type band and the mutant band are marked with arrows. Lane 1 contains 50 base pair ladders (Fig. 1).

4.2. Creatine kinase (CK) serum concentration

The mean CK serum concentration was 12.4 U/L (0.42) in healthy dogs and 184 U/L (0.34) in dystrophic dogs. Generally speaking CK values are <125 U/L in healthy dogs and cats [3,22].
4.3. IGF 1-serum concentration

All animals (healthy and dystrophic) presented an increase in IGF-1 levels from the first to the third month. During this period, healthy and dystrophic animals presented similar weight gains, which reflected a preclinical condition of GRMD animals, though GRMD presented IGF-1 values lower than non-dystrophic animals in all periods, (average IGF-1 values in the first 3 months: 332 ng/ml (0.25)) in dystrophic animals vs. 556 ng/ml (0.57); \( p < 0.05 \). After the first 3 months dystrophic animals presented a progressive decrease in IGF-1 levels that was mostly pronounced in severe clinical form subjects at the age of 7 months \( (p = 0.03) \). On the other hand, healthy animals presented a continuous increase in IGF-1 levels after the third month of life, though showing a different behaviour in IGF-1 concentration between animals after the fourth month, i.e. decrease (animal 5) or increase (animal 6), though the decrease observed in animal 5 was smaller than those observed in all dystrophic dogs (Fig. 2).

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Fig. 1. DMD genotyping. The GRMD mutation produces a novel Sau96I recognition site, and the digestion of the 310 bp genomic PCR is used to diagnose the mutant allele. The wild-type band and the mutant band are marked with arrows. Lane 1 contains 50 basepair ladders.

Fig. 2. The IGF-1 concentration and animal’s body weight are shown from month 1 to month 7 in healthy dogs (5 and 6) and in severe dystrophic dogs (1 and 2) and benign dystrophic dogs (3 and 4). All animals presented an increase in IGF-1 levels from the first to the third month. At the age of seven months, dystrophic animals presented a progressive decrease in IGF-1 levels at the age of 7 months. The body weight increase was progressive in all animals but less pronounced in the severe dystrophic dogs at 7 months.
4.4. Anatomy

In all dogs, healthy and dystrophic, the hypophysis was localised in the Sella turcica of the basisphenoid bone, adhered to the tuber cinerium in the infundibulum. Furthermore, the gland was placed caudally to the optic chiasm, optic tracts and caudally to cerebral peduncles and mamillary body. The gland was surrounded by dura-mater and composed by two parts, i.e. adenohypophysis which was divided itself into pars distalis and pars proximalis, and neuro-hypophysis. The adenohypophysis presented a yellowish–pinkish colour and was latero-rostrally oriented (Fig. 3).

The Hypophysis' length, width and thickness were 6.97 cm (0.01), 6.11 cm (0.04) and 4.14 cm (0.12), respectively, for healthy dogs and 6.63 cm (0.03), 6.48 cm (0.06) and 4.11 cm (0.09), respectively, for dystrophic animals. Differences between the groups were not significant \( p = 0.35 \).

4.5. Histology

4.5.1. Microstructure

Granulated cells were homogeneously distributed in clusters of 8–10 cell profiles (average of seven cell profiles) in the pars distalis of adenohypophysis in all animals. All clusters were located close to capillaries, though they appeared isolated in some cases (Fig. 4). Furthermore, cell shape varied between oval and spherical in both groups.

4.5.2. Immunohistochemistry

Immunofluorescence showed that GH-cells were distributed along the pars distalis of adenohypophysis in both groups, simply confirming what had been observed by bright field light microscopy in which these cells appeared granulated (Fig. 5A and B).

4.5.3. Ultrastructure

In granulated cells electron-dense granules were evenly distributed in the cytosol (Fig. 6A and B) and they had a double membrane (Fig. 6A1 and B1).

4.6. Stereology

4.6.1. Numerical density of GH-granulated cells, \( N_Y(GH\text{-cell/Hypo}) \)

In healthy dogs, the numerical density of GH-granulated cells was \( 93 \times 10^{-3} \text{mm}^{-3} \) (0.11) and \( 113 \times 10^{-3} \text{mm}^{-3} \) (0.10) in dystrophic animals. No significant differences were noted between the groups \( p = 0.13, \) Fig. 7).
4.6.2. Adenohypophysis volume, $V(Hypo)$

Adenohypophysis volume was 0.15 mm$^3$ (0.56) in healthy dogs and 0.14 mm$^3$ (0.52) in dystrophic animals. No significant differences were noted between the groups ($p = 0.89$, Fig. 7).

Fig. 5. GH-immunolabelled cells (white arrowheads) from adenohypophysis of a healthy (A) and a dystrophic (B) Golden Retriever dog. Scale Bar: 40 µm.

Fig. 6. Ultrastructure of the GH-granulated cells from the adenohypophysis of a healthy Golden Retriever (A, A1) and a dystrophic Golden Retriever dog (B, B1) showing electron-dense granules (larger white arrowheads) surrounding the nucleus ($N$) in A and B. Granules have a double membrane (thinner white arrowheads in A). Black arrowheads indicate plasma membrane in A. Figures A1 and B1 represent higher magnifications of A and B, respectively, showing details of granule ultrastructure such as their size (larger white arrowheads) and double membranes (thinner white arrowheads in A1). Scale Bar: (A) 500 nm, (B) 1 µm, (A1) and (B1) 200 nm.

4.6.3. Total number of GH-granulated cells, $N(GH-cell)$

GH-granulated cell number was 14,900 (0.64) in healthy dogs and 16,200 (0.49) in dystrophic subjects. Mean values were not significant between groups ($p = 0.87$) (Fig. 7).

4.6.4. Volume density of GH-granulated cells, $V_{V(GH-cell/Hypo)}$

In healthy dogs, the volume density of GH-granulated cells was 0.24 (0.04) and 0.39 (0.08) in dystrophic dogs. Differences between groups were significant ($p = 0.01$) (Fig. 8). The error variance for ratios (CE
ratios) was 0.02 for healthy dogs and 0.04 for dystrophic dogs.

4.6.5. Mean GH-granulated cell volume, $v_{N}$ (GH-cell)

In healthy dogs, the mean cell volume of GH-granulated cells was 2579 $\mu m^3$ (0.06) and 3415 $\mu m^3$ (0.06) in dystrophic dogs. Differences between groups were significant ($p = 0.01$, Fig. 8).

4.6.6. Volume density of GH-cells’ granules, $V_{V}$(GH granules/Hypo)

In healthy dogs, the volume density of GH-cells’ granules was 0.09 (0.46) and 0.12 (0.44) in dystrophic dogs. Differences between groups were not significant ($p = 0.23$). The error variance for ratios (CE ratios) was 0.05 for healthy dogs and 0.03 for dystrophic dogs.

Fig. 7. GH-cell numerical density, total number and adenohypophysis volume in dystrophic and healthy Golden Retriever dogs. There were no group differences in these data ($p > 0.05$). Horizontal bars indicate group means.

Fig. 8. GH-cell volume density and mean cell volume in adenohypophysis from dystrophic and healthy Golden Retriever dogs. There were significant group differences for both volume density and mean cell volume ($p < 0.05$). Horizontal bars indicate group means.
4.6.7. Total granule volume per GH-granulated cell, \( V(\text{granules}, G \text{ cell}) \)

The total volume of granules in an adenohypophysis’ GH-cell was 249 \( \mu m^3 \) (0.48) in healthy dogs and 415 (0.43) \( \mu m^3 \) in dystrophic dogs. Differences between groups were significant (\( p = 0.008 \)).

4.6.8. Total granule volume in all adenohypophysis’ GH-cells, \( V(\text{granules}) \)

The total volume of granules in all adenohypophysis’ GH-cells was 3.67 \( \times 10^6 \) \( \mu m^3 \) (0.55) in healthy dogs and 6.51 \( \times 10^6 \) \( \mu m^3 \) (0.69) in dystrophic dogs. Differences between groups were significant (\( p = 0.03 \)).

5. Discussion

The discussion will be pursued from the data obtained from relatively small sample size which was due to the very restricted laws to access Golden Retriever dogs in Brazil where animals cannot be euthanised for scientific purposes.

5.1. Anatomy

The hypophysis was placed ventrally to the hypothalamus being attached by means of the tuber cinereum inside hypophyseal fossa of the basisphenoid bone. This localisation was similar to that reported by Evans [35] and Dyce et al. [36]. There were no differences in the macroorganisation of the adenohypophysis between healthy and dystrophic animals, i.e. colour (yellow-pink), localisation (hypophyseal fossa) and size.

5.2. Histology

5.2.1. Microstructure

Granulated cells were homogeneously distributed in the pars distalis of the adenohypophysis in all animals. Cells were arranged in groups of seven cell profiles on average and were surrounded by capillaries as described in studies published by [37–39]. These authors have reported that GH-granulated cells are acidophilic in various species such as rats, pigs, dogs, humans and sheep. These cells were immunolabelled by means of specific markers for each species. In this present study, GH-granulated cells varied in shape, being usually spherical or oval.

5.3. Stereology

Inter-group differences in all stereological parameters were non-significant except volume density and mean GH-granulated cell volume as well as total granule volume per GH-granulated cell and total granule volume in all adenohypophysis’ GH-cells.

As for GH-granulated cell volume density, a 62.5% increase was noticed in dystrophic dogs as well as 32.4% increases in the mean GH-granulated cell volume.

Muscular dystrophy was characterized by a 32% increase in GH-cell volume which was accompanied by a 67% increase in the total granule volume per GH-granulated cell and a 77% increase in total granule volume in all adenohypophysis’ GH-cells.

It seems that during muscular dystrophy GH-granulated cells become bigger and occupy a larger fraction of the volume of the adenohypophysis without significant changes in either numerical density or total number of cells. In addition, a single GH-cell can afford as many as twice the granule volume when compared to a healthy GH-cell.

The increase in granule volume might be related to a reduction in the exocytosis process of GH-secretory granules leading to an accumulation of secretion granules within somatotrophs (GH-granulated cells) and causing an increase in both granule and cell size.

As reported for Duchenne muscular dystrophy in humans, GRMD is characterized by the absence of dystrophin which is a protein found in the musculoskeletal system and in neurons in specific regions of the central nervous system where it may cause alterations in the architecture of the SNC, dendrite abnormalities and reduction in the number of neurons in the brain stem and cerebral cortex in both humans and in laboratory animals [40].

Furthermore, the glandular part of the pituitary develops from Rathke’s pocket, derived from the oral plate. It begins to function as a secretory organ between 17 and 18 days of development. By 14.5 days the dystrophin probe gives a relatively strong signal over Rathke’s pocket [41].

Another striking site of dystrophin gene expression in the embryo is in the precursor cells that will form the glandular component of the pituitary. In this case, dystrophin may be playing a role in conjunction with the cytoskeleton in the secretory processes essential for function of the gland. Given the high level of dystrophin transcription in Rathke’s pocket, it is probable that pituitary function is perturbed when the gene is not expressed correctly, during formation of the glandular component of pituitary. In the light of the new localisation of dystrophin gene expression described here, we would predict that Duchenne patients may suffer from endocrine malfunction, from perturbations in circadian and seasonal rhythms, and from olfactory deficiencies [41].

As with Houzelstein et al. [41] we hypothesise that the absence of dystrophin in the adenohypophysis might alter peptide secretion patterns and their regulation, e.g. an accumulation of secretion granules within somatotrophs (GH-granulated cells) which become larger as well as their granules.
5.4. IGF-1 serum concentration

In humans, DMD may develop into two clinical forms: benign and severe according to the severity of symptoms [7]. In dogs, a similar clinical distinction is often made at the age of 7 months. IGF-1 was selected to investigate the characteristics of the GH/IGF-1 axis since it is more stable during the day and does not suffer large secretion oscillations as seen with GH [42,43,26].

In the present study IGF-1 concentration was both disease (presence, absence and severity of clinical form) and age-dependent which is in accordance with other studies already reported in the literature, e.g. in humans [44]. Furthermore, the decrease in IGF-1 concentration was more remarkable in dogs with a severe clinical form of GRMD at month 7. In contrast, a mild IGF-1 reduction was also observed in benign dystrophic animals.

As a suggestion for further investigations, we hypothesise that reduction in IGF-1 concentration in dystrophic dogs may be related to four main factors: (i) a lesser secretion of GH; (ii) a reduction in the number of GH-receptors in the liver; (iii) in the case of severe ill animals at 7 months it may be the result of an inadequate nutritional state and (iv) accumulation of secretion granules within somatotrophs (GH-granulated cells) as stated above.

6. Conclusions and remarks for future studies

Although unknown, a possible mechanism may be proposed as follows: absence of the dystrophin in the cytoskeleton would produce instability in the GH-granule membranes and as a result reduction in the exocytosis of GH-secretory granules. As a consequence, GH granules as well as GH-granulated cells become larger and now occupy a more significant fraction of the adenohypophysis volume, even though there are no significant changes in the total cell number.

Forthcoming investigations may focus on the dystrophin and its functional role in adenohypophysis’ GH-secretory granules.

Finally, and in contrast to earlier reports, our data suggest that a lower IGF-1 level may be more related to a severe, as opposed to a benign, clinical form of muscular dystrophy. Indeed, some authors have postulated that the benign clinical form of human muscular dystrophy is associated with GH deficiency, though GH was not directly determined since L-DOPA determination was used in those studies [7,8,33,34,45,46]. As a result, the same authors pointed out that GH inhibitors, such as mazindol, should be used to treat Duchenne muscular dystrophy [45,46]. In contrast, it has been reported that mazindol does not slow the progression of Duchenne dystrophy [47,48] and cause several adverse effects in boys after long term use [48]. The latter studies used IGF-1 determination, which more accurate than L-DOPA, more stable during the day and does not suffer large secretion oscillations as seen with GH [42,43,26].

As with [47,48], our data suggest that lower IGF-1 circulating levels are more related to a severe than to a benign clinical form of muscular dystrophy.

A very interesting therapeutic question to focus on in further studies would be whether GH inhibitors or GH imitators should be used in the treatment of either Duchenne or Golden Retriever muscular dystrophy.

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References


