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Microspectrophotometric Quantification of the Skeletal Muscle Glycogen Contents with Aging

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Abstract

Background Skeletal muscle fibers contain about 2% of its weight is glycogen, this glycogen used to keep the muscle functioning if it fails to receive sufficient oxygen. PAS stain is useful in detecting cytoplasmic accumulation of glycogen. Glycogen had been studied in skeletal muscles under various state of muscle activities and nutritional states but glycogen quantification with aging is not clearly defined till now.

Objectives Quantification of the mean glycogen concentration in skeletal muscles fibers stained with PAS stain in various age groups by microspectrophotometry.

Methods The tibialis anterior muscle of 20 Albino male rats (rattus rattus norvegious) of neonate, 3, 6, 9, 12, and 18- months were selected. Paraffin blocks were performed, sectioned and stained with PAS stain.
Analysis of the PAS stained sections by microspectrophotometry at 510 nm wave length. For the test group, mean absorbance, standard deviation, maximum, minimum, and mode values were estimated and compared with the control groups.

Results A significant difference in PAS absorbtion between test and control groups, and among different age groups, being increased with age.

Conclusion The variation in PAS absorbtion with aging indicates that the glycogen content in skeletal muscle increase with aging, this could be due to the influence of age on skeletal muscle glucose transport and glycogen metabolism.

Key words Skeletal muscle, PAS, Glycogen, Microspectrophotometry

Introduction

Muscle glycogen is an important fuel during exercise and its depletion is used as an indicator of fiber recruitment pattern during various types of exercises [(1)].

Although data related to muscle glycogen are interpreted as showing it is homogenous when quantified biochemically [(2)]. It is now well recognized that glycogen exists as individual particles located in distinct subcellular locations [(3-5)]. It's found in the sarcoplasm in form of coarse granules that seen in Electron microscope [(6-7)].

Glycogen in skeletal muscle can be demonstrated in paraffin sections by the periodic acid-Schiff (PAS) reaction [(8)], but conflicting reports exist concerning its validity as a quantitative test for polysaccharides [(9)].

A linear correlation between photometrically measured absorptions of PAS-stained tissue (paraffin embedded endometrium) and the

chemically measured glycogen content was reported and high correlation has been reported between glycogen concentration measured by microphotometry of single fibers of PAS stained cryostat sections and concentrations determined by biochemical analysis of human muscles [(10)].

In 1979 Halkjaer and Ingemann [(11)] described identical concentration of PAS (colour) in histological sections from the same human muscle irrespective of the type of preparation either cryostat or paraffin-embedded sections. The histochemical assessment of the glycogen content in various types of muscle fibers is usually carried out by subjective rating of the intensity of the PAS staining as described for animals [(12)], the subjective rating has been extensively used for human muscles [(13)].

Periodic acid-Schiff (PAS) stain when preceded with α-amylase treatment this will digest glycogen into smaller units that would be washed away during tissue processing and by comparing a slide stained by PAS technique with and without diastase digestion could reveal the amount of glycogen present in tissue [(11)].

However the glycogen concentration in skeletal muscle aging has not been thoroughly investigated. This study aim to demonstrate the effect of aging on glycogen concentration in PAS stained skeletal muscle sections by using microspectrophotometry.

Methods

- ***Animal sampling:***

A sample of twenty albino rats (Rattus norvegieus) male was selected, with different age: neonate (25 day), 3, 6, 9, 12, 18 month. Scarified animals killed with chloroform. Tibialis anterior muscle was selected for the study it taken out and divided into two halves and fixed in Bouins fixative.

- ***General histological preparation:***

Paraffin blocks were prepared and sectioned at 10µm thickness by Reichert-jung 3030-Biocutmicrotome.

- ***PAS –stain: (11).***

The paraffin sections were deparaffinized by xylene, rehydrated in descending concentration of ethanol alcohol 99.9%, 96%, 70% for 3minutes in each concentration, sections oxidized in 1% periodic acid for 5minutes, then treated with Schiff reagent for 10 minutes at 38°C, dehydrated in ascending ethanol alcohol 70%, 96% and 99.9% (3 minutes) for each concentration, cleared in xylene and mounted in Eukitt.

Control sections were preincubated in1% amylase (30 minutes, at 37°) before oxidation in periodic acid.

- ***Quantification of PAS staining intensity***:

Using microspectrophotometry, PAS staining intensity was expressed as absorbance and measured by spot measurement with a circular measuring diaphragm using Reichert-jung microspectro-photometer. Serial sections of each age group were scanned and 50 values recorded for each age group.

The wave length of the transmitted light was 510 nm selected as (Isobestic wave length), corresponding to the maximum absorption of the PAS-positive material [(11)].

The absorbance of each section measured by sum of absorbances of its fibers (cells), calculation of the mean of absorbance, standard deviation for both test and control groups.

In test group the maximum, minimum values of PAS stain absorption and mode (most frequent absorbance value) are calculated for each age group.

For the test group analysis of varience done by ANOVA (single factor) test to analyze the varience among the different age groups. Tukey's test which is usually refered to as HSD (honestly significant difference) used as a multiple comparison test that make use of a single value against which all differences in means are compared this value called HSD is given by:

HSD=qα, k, N-k√MSE/n

Where α is a chosen level of significant, k: number of means in the experiment, N: the total number of observation in the experiment, n: the number of observation in the treatment, MSE: is the error mean square from the ANOVA table, q: obtained by entering appendix table K with α, k, and N-k [14].

Technical setting of reichert-jung microspectrophotometry [15]:

It contains three units:

1. Voltage stabilizer unit by reichert-jung this should kept at 12 voltages through out the process of measurement.
2. Power unit.
3. control unit: data entry needed at this unit as:

A. Mode: extinction (optical density).
B. Isobestic wave length:510nm
C. Display rate :5 sec^{-1}
D. Damping rate: 10000 msec
E. Objective lenss:10X
F. Magnichanger of polyvar microscope:1.25X
G. Measuring diaphragm 10µm (numeric value 4) selected from the polyvar microscope.

Measurements of the control group:

Control group include sections from same age groups that treated with 1% amylase digestion prior to the step of Schiff reagent in the staining procedure, these slides used to detect the percentage of glycogen that lost by amylase digestion in each age group compared to test group. Student t-test (two samples with unequal varience) used to compare the difference in the mean absorption of skeletal muscle for the PAS stain between the test and control groups.

Results

PAS stain the skeletal muscle sarcoplasm homogenously pink in color not showing the myonuclei since in this procedure the Haematoxylin stain not used as a counter stain. It shows the connective tissue compartments of the muscle tissue: epimysium, permysium, and endomysium with the neurovascular bundle in the epimysium (Figures 1-3).

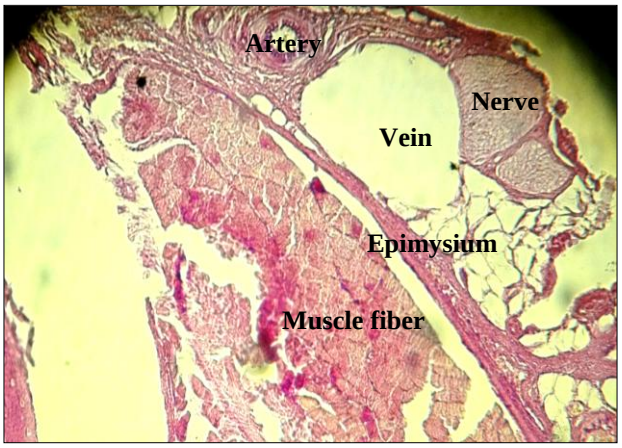

Figure 1. Section in neonate tibialis anterior shows the connective tissue covering (the epimysium) and neurovascular bundle (artery, vein and nerve). (40X)

The muscle fibers show various shades of pink color in same age group in single section indicating various rates of PAS absorbtion in different fiber groups (Figure 2).

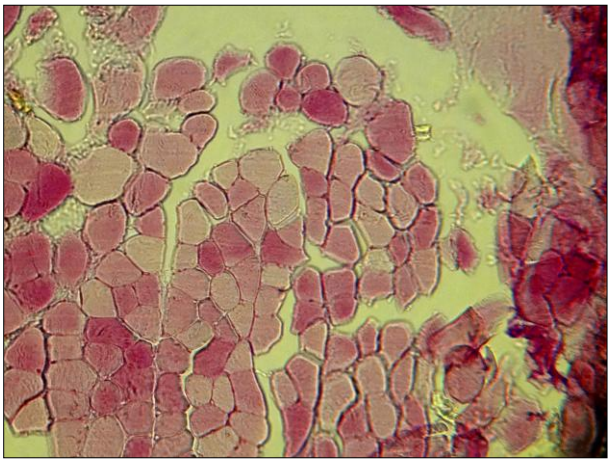

Figure 2. Section in tibialis anterior of 12-month showing fibers with homogenously stained sarcoplasm without myonuclei with various intensities of PAS stain in different fibers. (400X)

The absorbance of PAS stained material was determined by spot measurements and area scanning by systematic scanning of a fifty areas in each age group with the aid of illuminated

measuring diaphragm of the microspectro-photometry, the mean absorbance is considered the best representative for whole age group absorbance.

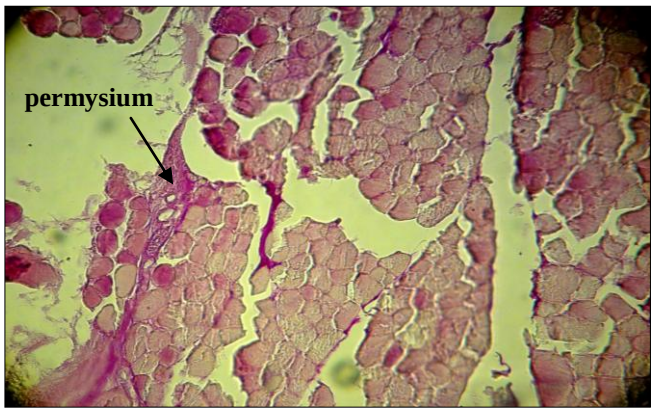

Figure 3. Section in tibialis anterior of 6-month showing the permysium of the skeletal muscle and muscle fibers without myonuclei. (400X)

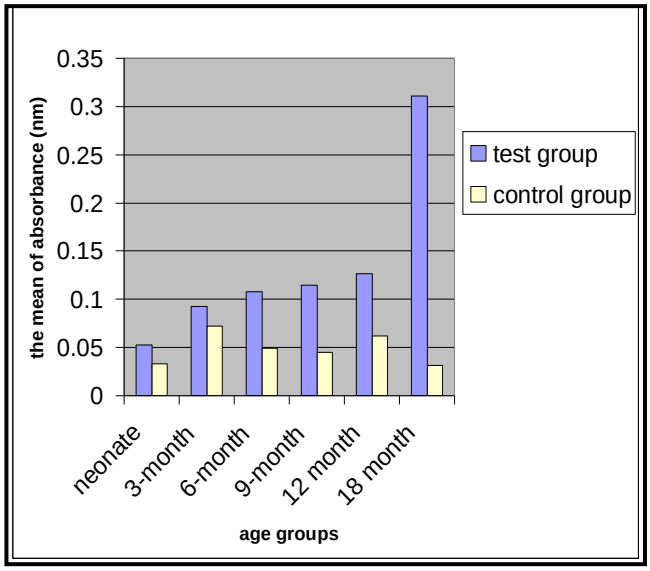

Figure 4. The mean absorbance of PAS stain (nm) measured by microspectrophotometry in test and control group for the six age groups.

The mean absorbance of control groups that treated with 1% amylase showed marked reduction from the mean of absorbance of test groups in all age groups, as shown in table 1 and figure 4.

Student t-test show statistically significant P – value: since t statistic > t critical table 3 indicating that a significant difference in PAS absorption found between test and control groups of the different age groups.

The timing and tissue thickness were fixed throughout the procedure; and all slides were stained in single container of Schiff reagent.

The mean absorbance of test groups increased with aging being in its lowest value in neonate age group and increased gradually reaching its highest value in 18- month age group (Figure 4 and table 1).

Maximum value of absorbance of PAS stain in a single spot were seen in 18- month age group, followed by 3-month age group, then 12-month age group, then 9-month age group, and lowest in neonate age group (Table 2).

Minimum value of PAS stain absorbance in single spot was recorded in 9-month age group, then in neonate, 3-month, 6-month, 12-month and 18-month consequently (Table 2).

The mode (most frequent observation) of absorbance of PAS in single spot show similar values in neonate and 18-month age group, and close values in 9-month and 6-month age groups (Table 2).

The analysis of varience among the age groups of the test showing a significant difference in PAS absorbance among the different age group, since F (calculated) **> F critical Table (4).**

Tukey's test used to demonstrate which age groups contribute to the significant overall p - value. In table 5, all age groups show a significant HSD value, in other words all groups contributed to the significant p-value.

Table 1. The mean absorbance of PAS-stain ± standard deviation measured in control and test samples in each age group.

Age groups	Mean value of maximum absorbance of test and control groups in fifty areas in each age group±SD (nanometer)	
	Test	Control
Neonate	0.05236±0.073119	0.033±0.02372
3-month	0.09296±0.049865	0.07208±0.097552046
6-month	0.10786±0.140265202	0.04926±0.033557
9-month	0.1144±0.146127	0.0448±0.02629
12-month	0.1265±0.181438	0.06198±0.017689
18-month	0.31056±0.086654	0.03178±0.017197

Table 2. The maximum, minimum and mode of PAS absorbance values in a single spot in each age group. (Nanometer)

PAS absorbance	Neonate	3-month	6-month	9-month	12-month	18-month
Maximum	0.54	0.198	0.86	0.77	0.99	0.455
Minimum	0.006	0.01	0.019	0.005	0.032	0.088
Mode	0.033	0.08	0.095	0.093	0.06	0.333

Table 3. t- Test for the comparison between the mean absorbance of the test and control groups.

t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	Variable 2
Mean	0.134107	0.048816667
Variance	0.008129	0.00025424
Observations	6	6
Hypothesized Mean Difference	0	
df	5	
t Stat	2.281709	
P (T<=t) one-tail	0.035693	
t Critical one-tail	2.015048	
P (T<=t) two-tail	0.071385	
t Critical two-tail	2.570582	

Table 4. ANOVA single factor test for the different age groups

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
Neonate	**50**	2.618	0.05236	0.005346		
3-month	**50**	4.648	0.09296	0.002486		
6-month	**50**	5.393	0.10786	0.019674		
9-month	**50**	5.72	0.1144	0.021353		
12-month	**50**	6.325	0.1265	0.03292		
18-month	**50**	15.528	0.31056	0.007509		

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	2.032322	5	0.406464	27.31351	1.09E-22	2.244703
Within Groups	4.375144	294	0.014881			
Total	6.407467	299				

Table 5. Demonstrate the significant HSD for the PAS absorption for each age group versus other groups.

Compared age groups	Difference in means	df	HSD	Significance
Neonate/3month	0.0406			significant
Neonate/6 month	0.0555			significant
Neonate/9 month	0.06204			significant
Neonate/12 month	0.074			significant
Neonate/18 month	0.2585			significant
3 month/6 month	0.0149			significant
3month/9 month	0.02144			significant
3 month/12 month	0.0339	5	1.3596	Significant
3 month/18 month	0.2176			Significant
6 month/9 month	0.00654			Significant
6 month/12 month	0.01864			Significant
6 month/18 month	0.2027			Significant
9month/12 month	0.0121			Significant
9 month/18 month	0.019616			Significant
12 month/18 month	0.18406			Significant

Discussion

PAS stain oxidize the hydroxyl (-OH) group that found in glycogen to aldehyde group (-CHO) by the periodic acid, this aldehyde group will react with the Schiff reagent to form the red-purple product, so PAS reaction is useful in detecting intracellular glycogen in skeletal muscle fibers. Haematoxylin stain is usually used as counter stain but not in this work [16].

The validity of PAS stain for assaying glycogen muscle contents was confirmed by a good correlation between the biochemical determined concentration in a whole muscle biopsy and the

mean concentration calculated from the area fractions for each principal fiber, and a high correlation was found between area scanning and spot measurements [10].

The absolute requirements for the use of PAS stain as a mean of obtaining quantitative expression of glycogen concentration must ensure that only glycogen in the tissue is stained and that's all glycogen or a constant fraction of the glycogen is present [17-18] .

These requirements have been fulfilled since most of the PAS positive material inside the skeletal muscle fibers is glycogen that removed selectively with diastase digestion (α-amylase) digest glycogen into smaller units that would be washed away during processing and by comparing a slide stained by PAS technique with and without diastase digestion could reveal the amount of glycogen [11].

The differences in the mean absorbtion of PAS stain between the test and control samples shown in table 1 and table 3 caused by incubation of control sections in 1% amylase caused a reduction in the absorbance, which indicate that glycogen alone is responsible for the intracellular absorbance of PAS stained material [11].

Thickness of sections were fixed at 10µm and the timing of staining in periodic acid also fixed although no statistical differences was found between the PAS staining intensity (absorbance/thickness) in relation to either to change in thickness or the time of oxidation in periodic acid . This indicates that the oxidation in periodic acid for glycosyl of glycogen has reached a constant level during the first 5 minutes [11].

The differences in the activity of different Schiff reagents is another important factor which might seriously affect the PAS staining intensity this may be due to variation in the manufacture of the reagent or the way of its storage, so its important there for sections that to be compared are stained in same histochemical bath [11].

The variation of PAS stain absorption with aging could be due to the influence of age on skeletal muscle glucose transport and glycogen metabolism. There is an age-related alteration in skeletal muscle carbohydrate metabolism; muscle glycogenolysis is accelerated in old male rats compared with young animals, perhaps secondary to the age-related reduction in muscle oxidative capacity and blood flow [19].

In a study of perioral muscle specimens, lipid pigment (Lipofuschin) granules were present in 68.5% of the cases. These granules were PAS positive, they also stained with other stains :stained black or brown with the Masson-Fontana procedure, black with Sudan black and strong purple-pink with Ziehl-Neelsen staining; yellow auto fluorescence was emitted in ultraviolet light. Statistical analyses indicated a direct correlation between increase in quantity and distribution of the pigment and increase in age in both males and females [20].

Variation in minimum, maximum, and mode values of the test group in different age groups may be due to the type of muscle fiber on the spot on which the measuring diaphragm placed. In this study tibialis anterior muscle was selected since its mixed type of muscle with equal proportion of type I and type II fibers since the mean glycogen concentration was found different in different types of fibers , its higher in type II fibers than in type I fibers in resting muscles [21].

Training enhances muscle oxidative capacity and promotes muscle glycogen sparing during exercise by young and old rats, exercise training increases the muscle glycogen levels of older people. After one bout of exercise, muscle sensitivity for insulin-stimulated glucose transport is improved in young and old rats. These findings indicate that several age-related changes in muscle carbohydrate metabolism can be minimized by acute or chronic exercise [22].

Muscle glycogen recovery is the process through which the muscles of the body are replenished

with carbohydrate sources that have been depleted through the energy expended in exercise. Most glycogen is entirely consumed from muscle stores within 15 minutes to 30 minutes from the commencement of the exercise; the athlete may exhaust all of the stored glycogen reserves with 10 minutes of muscle effort. The glycogen must be recovered and the supplies restored at the conclusion of the activity. The primary danger associated with this depletion is damage to cells and muscle structures; it may trigger the breakdown of cell structures to create an alternative energy supply. Muscle glycogen depletion also places significant stress on the overall function of the immune system, such depletion, if not corrected, carries with it risks to the structure and the function of the body. Muscle glycogen depletion is most effectively counteracted through diet; athletes who understand the demands placed on their muscle glycogen stores will plan how they shall achieve glycogen recovery through the foods ingested before, during, and after their workouts and their competitive events [23-24].

On other hands Calorie restriction's (CR) effects on age-associated changes in glycogen-metabolizing enzymes were studied in rat tibialis anterior (TA) muscles in old (24 months) compared to young (6 months) rats maintained ad libitum on a standard diet. Age-associated impairments in Glycogen synthesis (GS) protein and activation-phosphorylation were shown in TA, but glycogen phosphorylation (GP) was inactivated in TA with age. CR did not alter GS or GP activity/protein levels in young rats. CR hindered age-related decreases in GS activity/protein. Thus, the predominant age-associated impairments on skeletal muscle and CR can attenuate the loss of GS activity/activation and stimulate glycogen accumulation [25].

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