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ESTIMATION THE LEVELS OF CONTRACTILE AND COLLAGENOUS PROTEINS OF BACK SKELETAL MUSCLE DURING FETAL DEVELOPMENT IN RATS

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ABSTRACT
Biochemical determinations of contractile and collagenous proteins made on the skeletal muscle tissues of rat fetus and new born rats. The biochemical change of contractile and collagenous proteins of muscle tissue that takes place during the intrauterine life is an essential part in the development and organization of skeletal muscles. The study was performed on the albino rat, as a mammalian model. The specimen of muscle tissue from 29 fetuses at different age starting from day 17 and 5 new born rats was including in this study. The biochemical assays were performed at the medical city teaching laboratories/ Baghdad. The estimation levels of contractile and collagenous proteins in the tissue sample were done by using Double Beam Spectrophotometer. As a result, there was an increase in tissue level of contractile proteins begin even before fetal life and also there was decrease in collagenous proteins that start during fetal life .It was concluded, the relative proportion between contractile and collagenous proteins in developing muscle tissue is inverted in new born rats when compared with those rats at day 17 of embryonic development.

KEY WORDS: Skeletal Muscle, Development, Contractile proteins, collagenous protein

INTRODUCTION

Proteins are the basic material of tissue structure**,** They are the most important component of striated skeletal muscles, their classification is correlated with the histological structure of muscle tissue .Muscle tissue is a composite tissue which is composed of, muscle cells (muscle fibers) specialized for contraction, connective tissue, nerve fibers and blood vessels [1]. Muscle growth occurs during embryonic development and continues in adult life as regeneration [2,3]. During embryonic muscle growth and regeneration in mature muscle, single nucleated myoblast fuse to each other to form myotubes, within the myotubes, bundles of contractile proteins are organized into myofibrils giving the tissue a striated appearance [3]. The myofibrils are basically composed of two filamentous, contractile proteins, actin and myosin. Contractile proteins are an important cellular component, critical to maintaining cell shape, mass, and other cellular functions[4]. The mass of a muscle is made up of 75% water and more than 20% protein. The muscle proteins can be divided into contractile, regulatory, sarcoplasmic and extracellular forms; the most important are the contractile proteins actin and myosin[5]. The contractile proteins was first shown by [6] and include troponin, tropomysin, M-protein, beta actin, gamma actin, C–protein which are of great importance. Sarcolemma is also protein component, and others proteins such as elastin, collagen and reticulin are also found in the muscle, there are also myoglobin, and others[5,7]. Striated myofibers held together by connective tissue, the muscle connective tissue layer is composed of collagen and elastine, but collagen is the more abundant of these two proteins, representing about 2% to 6% or more of dry weight of muscle [8,9]. This layer contains and supports the contractile tissues of the muscle and at its extremities provides the connection to the skeleton, connective tissue of muscle is almost as important as the muscle fibers [10] for without it there would be no structure to the muscle belly and no way for the movement and forces produced in the fibers to be transmitted to the tendon. There are variations in amount of collagen between different muscles of the same animal [11].

MATERIALS AND METHODS

The study was performed on the albino rat (*Rattus rattus norvegicus albinus*), as a mammalian model. Male and female couples were kept together in mating cages .A careful examination of the cage was done each morning and the presence of vaginal plug was considered an indication of copulation. The day when the vaginal plug was found was considered as “day one post coitum” (dpc), the occurrence of vaginal plug considered as the first day of pregnancy .Pregnancies were dated by appearance of a copulatory plug. In this study we used the E-designation **[12]**.This method is used to standardize the embryological material, although this designation includes several parameters in combination.

This parameter includes, dpc, Witschi stage, Thieler’s stage. The day plug was found being E1.The subsequent days were sequentially numbered. At each dpc, starting from day 17 to day 21, two to three pregnant rat females were anesthetized to obtain embryos of that conception age, the embryos were washed with normal saline and delivered into Petri dishes, and the embryo was examined under magnifying lens for features of staging. Two pregnant rat females were allowed to complete pregnancy and have normal vaginal delivery. The new born litters were immediately separated from their mother and included in this work. Witschi staging system**[13]** was

correlated with the Theiler's stages [14], based on diagnostic features of the rat embryos. All rat embryos were sacrificed humanly by general anesthesia before obtaining muscle tissue sample. For tissue samples employed in biochemical assay, back skin was incised, carefully opening from occipital region caudally to the pelvic region. The muscles of the back were cleaned off subcutaneous material; the muscles around the vertebral column. The muscles were easily identified by their pinkish color, location and texture .Using blunt dissection by forceps and with aid of a probe, the muscles were teased away from the vertebral column and the skin was peeled away. The tissue sample was eventually freed away from its place by sharp dissection. The numbers of specimen for biochemical study are 29 and 5 new born rat. The tissue samples were trimmed under dissecting microscope to ensure inclusion of relevant parts of the sample. A small piece of back skeletal muscle tissue was first weighted then minced by chopping using a sharp razor blade. The minced pieces were put into appendrofetube, containing normal saline (50mg wet tissue per 1 ml of normal saline).The biochemical assays were performed at the medical city teaching laboratories/ Baghdad, 2010.

The biochemical assays for determinations contractile proteins were made according to Lowery assay [15], whereas collagenous proteins determined according to Reddy and Enwemeka assay [16].

Lowry Method for Contractile Protein Determination

The Lowry assay[15], is an often cited general use protein assay. For a long time it is the method of choice for accurate protein determination for tissue and cell fractions.

Principle

Under alkaline conditions, the divalent copper ion (Cu^{+2}) forms a complex with peptide bonds of proteins, and it is reduced to a monovalent copper ion (Cu^{+1}). Monovalent copper ion (Cu^{+1}) and the radical groups of tyrosine, tryptophan and cysteine react with Folin reagent. This is an oxidation reduction reaction, in which the folin reagent is reduced to heteropolymolybdenum blue and oxidation of copper and aromatic acids.

The Lowry method is sensitive to low concentrations of protein.

Lowry method is sensitive to pH changes and therefore, the pH range of assay solution should be maintained at 10-10.5. However, if the assay involves using very small volumes of sample, there will be little or no effect on pH of the reaction mixture.

Procedure

Reagents

1. Reagent A

It consists of 2mg sodium potassium tartarate , hydrated (4H20) (BDH); 100mg sodium bicarbonate (BDH); 500 ml 1N sodium hydroxide (BDH); water is added to one liter.

This will give (7mM Na-K tartarate, 0.81 sodium bicarbonate, 0.5N NaOH) final concentration. This reagent keeps for 2-3 months

2. Reagent B:

It consists of 2 mg sodium potassium tartarate, hydrated (4H20) (BDH); 1g copper sulphate (CuSO4.5H20) (Fluka), 90 ml H2O, 1N sodium hydroxide.

This will give (70mM Na-K tartarate, 40mM copper sulphate) final concentration. This reagent keeps for 2-3 months.

3. Reagent C:

It consists of one volume of Folin reagent (BDH) diluted with 15 volumes water.

4. Bovine serum albumin standard: (1.0 mg/ml).

Assay

1. A series of dilutions was prepared from bovine serum albumin standard
   l.0 mg/ml, to give solution that concentrations 0.3-1.0 mg/ml.
2. Ads 1.0 ml of each dilution of the standard, protein containing unknown (test samples), to 0.9m1 of Reagent A in a separate test tubes and mix.
3. Incubates the tubes for 10 minutes in water bath at 50°C, and then cool to room temperature.
4. Ads 0.1ml Reagent B, mix, incubates for 10 minutes at room temperature.
5. Rapidly adds 3.0 ml Reagent C to each tube, mix, incubate for 10 minutes in water bath at 50°C, then cool to room temperature. The final assay volume is about 5.0 ml.
6. Measures absorbance at 650nm in 1cm cuvettes using Centre 5 UV-Vis Double beam spectrophotometer.

Analysis

Standard curve of absorbance versus micrograms protein (or vice versa) and determines and the amounts of protein in test sample was obtained from the curve.

Reddy and Enwemeka Assay for Hydroxyproline Determinatio

The simplified method for analysis of hydroxyproline in biological tissue developed by Reddy and Enwemeka[16] , was used to quantify the collagen content through hydroxyproline determination.

Principle

The method is based on alkaline hydrolysis of the tissue homogenate and subsequent determination of free hydroxyproline in the hydrolyzates. Chloramines-T was used to oxidize the free hydroxyproline for production of pyrrole. The addition of Ehrlich's reagent resulted in the formation of a chromophore that can be measured at 550nm. The method is highly sensitive and reproducible when used to measure the amino acid in tissue homogenate.

Procedure

Assay

1- Prepares standard collagenous protein (mammalian, Fluka) samples (0, 2.4.6.8.10µl) and test sample of tissue homogenate (l0µl, 50 mg wet tissue/ml saline) in high temperature polypropylene tubes of 2ml capacity.

2- Adds 50µl of 2N sodium hydroxide (BDH) to each tube of standard and test samples and mixs at room temperature for 20 minutes

3-The tubes are hydrolysed (alkali hydrolysis in 2N NaOH) by putting on open flam fire of benzene burner for 20 min.

1. Chloramines-T (BDH) 50ul is added to each tube and mixed with the hydrolyzate; oxidation is allowed to proceed for 25 min at room temperature.

2. 50µl of 1M Ehrlich's reagent (BDH) is added to each tube and mixed, then incubated for 20 min at 65°C water bath.
3. Finally, the absorbance of the samples is read at 550nm in 1cm cuvattes using Centro 5 UV-Vis Double Beam spectrophotometer.

Analysis:

Prepares standard curve of absorbance versus micrograms protein (or vice versa) and determines the unknown amounts from the standard curve.

RESULTS

Each of the experimental muscle tissue was observed to undergo a significant (P [ANOVA]).The evaluation of the levels of contractile proteins in tissue samples forms the foundation of the biochemical part of this work. The estimation is all standardized as µg /ml of wet tissue homogenate obtained from (34) tissue samples recovered from rat embryos and new born litters.

Contractile proteins

The result showed that there is a highly statistical significant among all embryonic stages [P (ANOVA) <0.001].

The highest mean value of contractile proteins was 577.00 µg/ml of wet tissue homogenate of skeletal muscle found in group of the new born rat. While the lowest mean value of contractile proteins was 356.33 µg/ml wet tissue homogenate found in E17stage as it is shown in Table (1), Fig. (1).

TABLE 1: Descriptive statistics for contractile proteins contents of skeletal muscles tissue, value are in µg/ml of wet tissue

Embryonic age	Variables	µg/ml of Wet Tissue Homogenate	P(ANOVA)
E17	Mean	356.333	0.001<
	SD	3.777	
	N	6	
E18	Mean	376.833	
	SD	2.639	
	N	6	
E19	Mean	446.000	
	SD	4.359	
	N	5	
E20	Mean	496.714	
	SD	0.951	
	N	7	
E21	Mean	554.200	
	SD	2.588	
	N	5	
New born rats	Mean	577.000	
	SD	4.183	
	N	5	

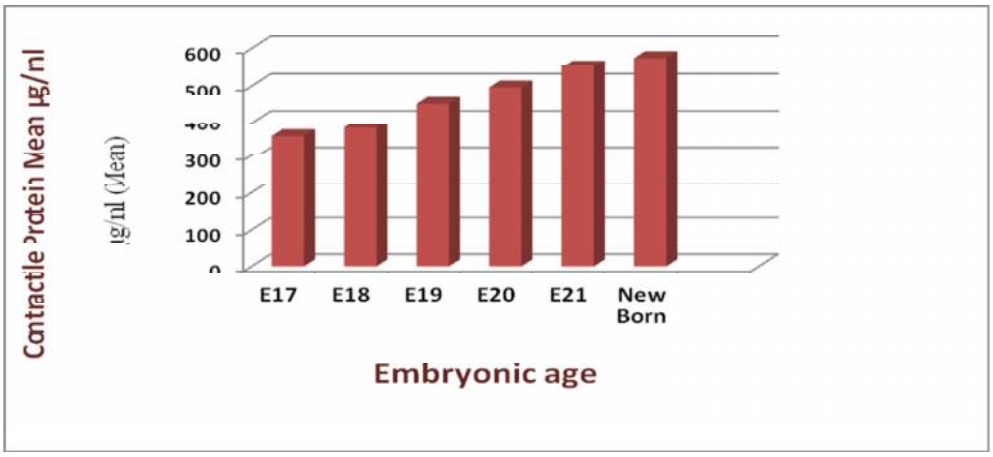

FIGURE 1: Histogram of Mean Values of Contractile Proteins Estimated at Various Embryonic Stages and New Born Rats.

Collagenous protein

The results showed that there is a highly statistical significant among all embryonic age [P (ANOVA) <0.001].

The highest mean value of collagenous proteins was 5.07 µg/ml of wet tissue homogenate of skeletal muscle at E17 Whereas the minimal mean value of collagenous proteins estimated was 3.83 µg/ml of wet tissue homogenate in new born rats as it is shown in Table (2), Fig.(2).

The biochemical results shows that the ratio of contractile proteins concentration to callagenous protein concentration estimated by µg/ml of wet tissue homogenate in the back muscles of rat fetus at E17 was (**1:2)**, as shown in (Fig.3).

This ratio is inverted in new born and becomes (**2:1)**,as shown in (Fig.4).

TABLE 2: Descriptive statistics for collagenous proteins content of skeletal muscle tissue, value are in µg/ml of wet tissue Homogenate

Embryonic age	Variable	µg/ml of Wet Tissue Homogenate	P(ANOVA)
E17	Mean	5.073	0.001<
	SD	0,059	
	N	6	
E18	Mean	4.867	
	SD	0.103	
	N	6	
E19	Mean	4.332	
	SD	0.024	
	N	5	
E20	Mean	3.749	
	SD	0.043	
	N	7	
E21	Mean	3.758	
	SD	0.088	
	N	5	
New born rats	Mean	3.826	
	SD	0.043	
	N	5	

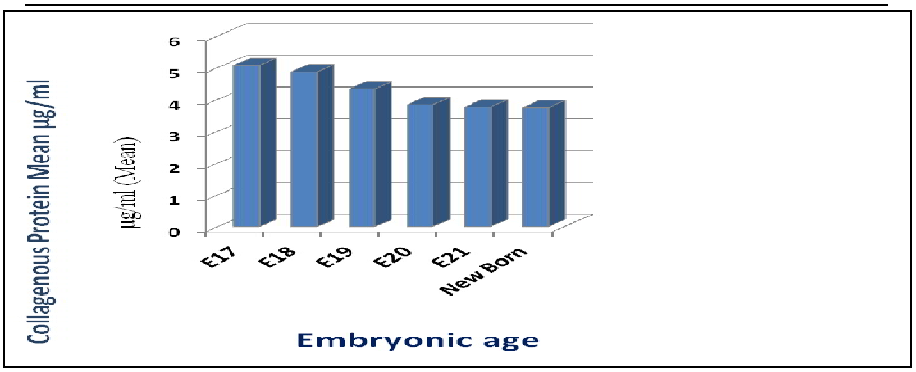

FIGURE 2: Histogram of Mean Values of Collagenous Proteins Estimated at Various Embryonic Stages and New Born Rats

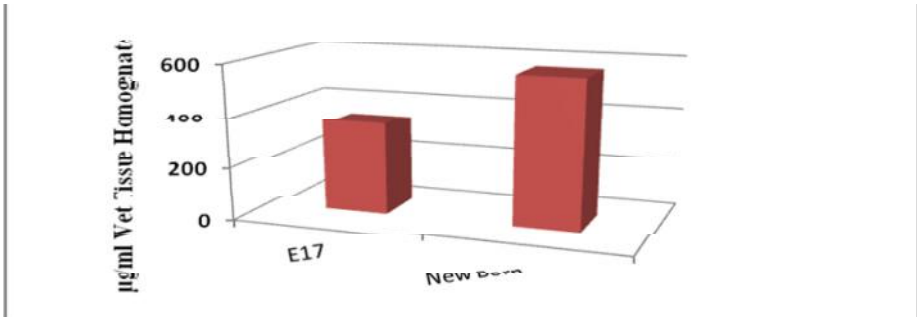

FIGURE 3: Bar Diagram Compares The levels of Contractile Proteins Estimated in Skeletal Muscles of Rat Embryos at E17 and that of New Born Rat

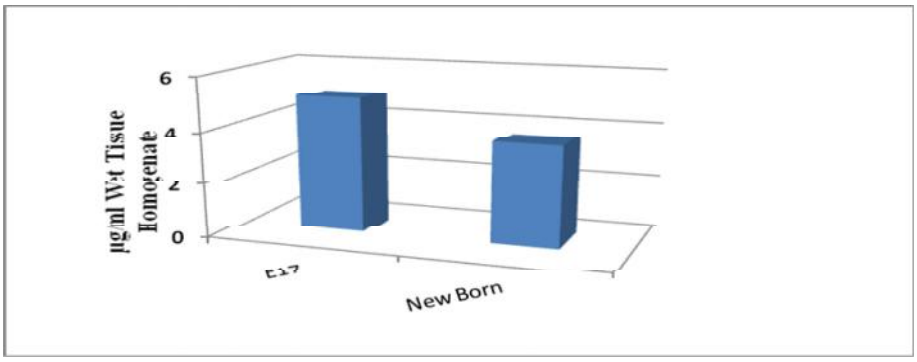

FIGURE 4: Bar Diagram Compares The levels of Collagenous Proteins Estimated in Skeletal Muscles of Rat Embryos at E17 and that of New Born Rat.

DISCUSSION

This study found that there is increase in the concentration of contractile proteins starting before day 17 and continuous till birth and postnatal life. The observations in this study on contractile proteins during skeletal muscle differentiation of the rat embryo confirm the reports of others made on chicken embryos by [17, 18, 19] reported the presence of myofiliment (myosin and actin) in the sarcoplasm of young myotube and *suggested that only actin involved in the* movements of the early myoblast and its appendages,they show that thick filament are not seen in mammalian myoblast. This finding agree with [20])who described the sequence in which the special proteins of muscle appear during embryogenesis which include the first protein appear is desmin, and then follows titin, muscle specific actin, myosin heavy chains and last nebulin at E11. According to the findings concluded by [21] who revealed that further growth of these fibers in width and length occur during fetal life was due to increasing rate of contractile protein synthesize and number. This observation explains the increasing of the muscle mass in the present experimental study.

Collagen content of skeletal muscle was determined via measurement of the collagen-specific amino acid, hydroxyproline (HYP), which is found almost exclusively in collagen and provides a direct measure of collagen content [22]. The concentration of HYP was quantified firstly by [23].

The studies of [24] and [25] have confirmed their original observations that embryonic collagen contains two genetically distinct collagens, designated types III and I.

According to the findings of [26] and [22] the collagen molecules are synthesized, then secreted from the cell into the extracellular space, cross linking between the collagen microfibrils is initiated and larger diameter fibrils form.

The biochemical results shows that the ratio of contractile proteins concentration to callagenous protein concentration estimated by µg/ml of wet tissue homogenate in the back muscles of rat fetus at E17 was (**1:2)**, as shown in (Fig.3).

This ratio is inverted in new born and becomes(**2:1)**,as shown in (Fig.4).The critical period of concentration inversion for each contractile and collagenous protein occur during the period E19-E21. This is a critical period in skeletal muscle development since during this period maturation of muscles takes place. There is no evidence of change in a biochemical value between E21 and new born rats, but there was clear evident change from E17 to E20.

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