THE EFFECT OF STRESSFUL EXERCSE AND / OR LOCAL IMPLANTS OF LABELLED BUFFY COAT CELLS ON MUSCLE REGENERATION

Nawfal K. Al-Hadithi* Ph.D.
Fara'id Y. S. Haddad,* Ph.D.
Hani T. Al-Azzawi,* Ph.D.

SUMMARY:

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Background: *The process of muscle regeneration is an importnant field in scientific work. Thus, an attempt has been carried out to find the link between the most important factors which may affect this process and these are exercise and local buffy coat implantation.*
Materials and methods: *168 male albino rats were divided into 5 groups. All of these groups were exposed to unilateral calf muscle injury of their right legs. The 1st group was regarded as control , the 2nd was exposed to stressful exercise , the 3rd was exposed to local implantation of labelled autogenous buffy coat cells at the site of muscle injury , the 4th was exposed to both events while the 5th was exposed to local injection of the labelling material at the injury site. Follow up of the process of muscle regeneration had been carried out for 28 days.*
Results: *Results have shown that muscle regeneration was enhanced in the 2nd and 4th groups where stressful exercise had been performed , possibly by providing myoblast precursors from the circulation and / or increasing myoblast spillage into the area by multiple fiber disruptions . Buffy coat had also enhanced muscle regeneration by transformation of some of them into myoblasts as indicated by the presence of labelled cells in the regenerated muscle tissue in the 3rd and 4th groups.*
Conclusions: *These findings had led to the conclusion that, muscle regeneration can be improved by using stressful muscle exercise together with local implantation of buffy coat cells at the site of muscle injury. An intimate relation had been predicted between buffy coat implantation, stressful exercise and muscle regeneration.*
Key-words: *Muscle injury, Muscle exercise, Muscle regeneration, Buffy coat cells*

Introduction

The role of cells , other than local cells , in muscle regeneration was first studied by Bayliss & Sloper in 1971[1]. They confirmed that exogenous cells from the circulation become myogenic progenitors on need. Studies in this field are going on using exogenous elements that might enhance myogenesis if applied at the site of injury like liver mince[2], bone marrow implants[3], and circulating leucocytes[4]. The effect of stressful exercise on the circulating leucocytes such as leucocytosis[5-8] with differential monocytosis especially of the early differentiated monocytes which have the ability to transform into other cell types, had raised in mind that the foci of exercise-induced muscle fiber disruptions could call for exogenous cells, and the easiest and quickest source which can offer these cells is the blood and the early formed monocytes could fit the required cell type[8]. The well known theory of demargination leucocytosis after exercise did not exclude the possibility that part of this leucocytosis is called by the injured muscle fibers themselves to supply them with muscle forming cell precursors. These facts initiated the idea that a link is present between these parameters (exercise, leucocytosis and muscle injury) but it seemed that true evaluation and verifying this link in a clear way in vivo had not been done in a separate scientific work yet. Hence the present study had been devoted to do so. Thus, statistical studies on the effect of stressful exercise and / or local implantation of leucocytes on regeneration of injured muscles were performed for the first time. In addition, an attempt was made to recognize the cell type of the buffy coat that might participate in this regenerative process.

* *Department of Anatomy, College of Medicine, University of Baghdad*

MATERIALS AND METHODS:

168 healthy male albino rats were obtained from the Animal Breeding Center of the College of Medicine, University of Baghdad. Their average

age was 22-24 weeks and their average weight was 300-330 grams. They were breeded in the same environment at 20-25° C room temperature with maintenance of diurnal lighting and fed with a vitamin supplemented diet and tap water on need. They were divided into five groups all of which were exposed to right calf muscle crush injuries. The 1st group (21 rats) was regarded as controls . The 2nd group (21 rats) was exposed to stressful muscular exercise by leaving the animal swimming freely in a swimming pool until it is severely exhausted taking about 70-90 minutes[9]. The 3rd group (42 rats) was subdivided into two subgroups of 21 animals each , one of which was injected at the site of muscle injury with autogenous buffy coat cells labelled with methylene blue (MB) by incubation with it for two hours at 37° C and the other was injected by buffy coat cells labeled with imferon by incubation for 3 hours[8,10]. The 4th group (42 rats) was dealt with as the previous one but after exercising the animals as in the 2nd group. The 5th group (42 rats) was also subdivided into two groups one of which was injected directly with MB at the injury site and the other was injected with imferon. Animals were sacrificed at days 1, 3, 5, 7, 14, 21 and 28 after the event as follows: Under light ether anaesthesia, animals were perfused with a freshly prepared 2% paraformaldehyde solution under pressure until complete fixation was achieved indicated by animal pallor and rigidity. The injured muscle was removed and divided into two parts one for longitudinal and the other for cross sections, quenched with liquid nitrogen and trimmed into 8-10µm sections in a cryostat. Sections of the 1st and 2nd groups were stained with routine H & E stain while those of the 1st subgroup of the remaining three groups were further divided into two groups, the 1st stained with nuclear fast red (NFR) while the 2nd stained with methylgreen (MG) stain. Sections of the 2nd subgroup of the remaining groups were stained with Perl's prussian blue stain. Eight slides from each animal were studied, four carrying LS and four CS, but the counting method was done for longitudinal sections only. In each slide, five fields of X400 magnification power were counted, four of them in the transitional area between the healthy and necrotic zones and the fifth in the center of the necrotic zone[2].

T test statistics was applied to evaluate the significance of difference between experimental and control groups.

RESULTS:

DAY 1: The severed ends of the muscle fibers showed eosinophilic staining in control group and were invaded by inflammatory cells which were mainly lymphocytes and concentrated in the center of the necrotic zone .The 2nd group showed similar findings with significant potentiation of the inflammatory reaction, increase in neutrophil percentage and slight increase in monocyte percentage. In the 3rd group, labelled leucocytes were demonstrated in the area, MB labeled cells were better demonstrated than imferon labeled cells, and for MB labeled cells, sections stained with NFR showed better tissue-stain contrast than those stained with MG. In the 4th group, a more intense inflammatory reaction was noticed compared to the previous groups, while sections of the 5 group were similar to those of controls apart from the appearance of good percentage of labeled leucocytes with extracellular deposition of the label.

DAY 3: Myoblasts were the regenerating markers in this stage. They were identified according to their histological appearance as oval or fusiform cells with abundant basophilic cytoplasm and large oval nucleus. Labeled myoblasts were seen in the last three groups with variable label intensity being most in the 4th group (Figure 1). Table (1) shows the counting results of this marker in all groups.

DAY 5: Free myoblast count was reduced and the regenerating markers in this stage were myotubes which are longitudinally arranged and fused myoblasts to form elongated synscytia. Early formed myotubes were predominating in this stage and labeled myotubes were seen in the last three groups with variable label intensity being most in the 4th group as shown in Table (2) & Figure (2).

DAY 7: The field is mainly occupied by myotubes which were more mature than those of day 5 having more nuclei, less basophilia in the cytoplasm and wider diameter. Labeled myotubes were seen in the last three groups being most intense in the 4th group. Table (2) shows the counting results of this marker and figure (3) shows their morphology.

DAY 14: Regenerating muscle fibers were seen in this stage occupying the field with some fibrous tissue among them. Labelled

fibers were seen in this stage in the last three groups (figure 4) and their counting results were shown in table (3.) together with the those of the following two stages.

DAY 21: Regenerating fibers showed more maturity signs with wider diameter, more eosinophilic cytoplasm, less centrally located nuclei and clearer striations. Labeled fibers were seen in the last three groups and was especially evident in the 4th group (figure 5).

DAY 28: Almost mature fibers were seen, with some labeled fiber nuclei in the last three groups, more in the 4th group (figure 6).

DAY	GROUP	MEAN COUNT	% OF MB-LABELLED CELLS	% OF IMF.-LABELLED CELLS
3	1	350± 14.719		
3	2	*490± 20.396		
3	3	#385± 12.288	10	5
3	4	*500± 9.128	17	9
3	5	375± 8.655	30	26
5	1	100± 4.795		
5	2	*81± 6.480		
5	3	#90± 6.701	4	2
5	4	*70± 4.991	6	3
5	5	105± 5.802	20	9
7	1	65± 4.787		
7	2	*40± 6.88806		
7	3	#60± 6.027	1	0
7	4	*30± 4.031	2	0
7	5	75± 3.774	6	2

TABLE 1 : Counting results for myoblast means in days 3 , 5 , & 7 of muscle regeneration with the standard deviation , * p value < 0.001 , # p value < 0.005 Counting was done under X400 magnification power

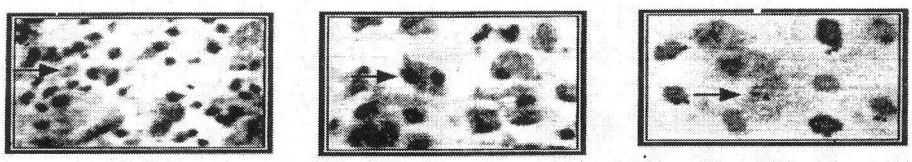
DAY	GR.	MEAN COUNT	NO. OF NUCLEI			% OF LABELLED MYOTUBES		% OF LABELLED NUCLEI/ MYOT.	
			5>	5-10	10 <	MB	Imf.	MB	Imf.
5	1	8 ±1.825	76	24					
5	2	*9 ± 1.414	69	31					
5	3	#8.5 ± 1	65	35		24	12	15	14
5	4	*11 ± 1.615	58	40	2	33	25	25	22
5	5	8 ±1.414	70	27	3	50	35	22	20
7	1	12 ±1.414	61	39					
7	2	*14± 0.975	46	49	5				
7	3	#13 ± 2.061	55	42	3	15	6	15	10
7	4	*16 ± 1.154	40	51	9	12	9	22	18
7	5	11 ±1.414	59	38	3	25	25	30	27

TABLE 2 : Counting results for myotube means in days 5 & 7 of muscle regeneration with the standard deviation , * p value<0.001 , # p value <0.005 Counting was done at X400 magnification power

DAY	GRP.	MEAN COUNT	NUCLEAR POSITION		% OF LABELLED FIBERS		% OF LABELLED NUCLEI/FIBER	
			Cent.	Peri.	MB	Imf.	MB	Imf.
14	1	17 ±0.975	35	65				
14	2	*19 ± 1.825	22	78				
14	3	#18 ± 1.154	30	70	15	10	20	16
14	4	*21± 0.816	19	81	20	12	27	18
14	5	17.5 ± 1.914	36	64	35	24	40	30
21	1	13 ± 0.816	16	84				
21	2	*15± 2.449	11	89				
21	3	*14.5± 1.258	18	82	13	8	18	12
21	4	*17± 2.100	7	93	16	10	21	16
21	5	#14± 0.957	15	85	30	20	35	25
28	1	11 ± 1.414	7	93				
28	2	*13 ± 1.414	3	97				
28	3	#11.5± 1.732	5	95	10	6	14	9
28	4	*13.5± 1.707	2	98	12	9	16	11
28	5	11 ± 1.414	9	91	20	13	24	17

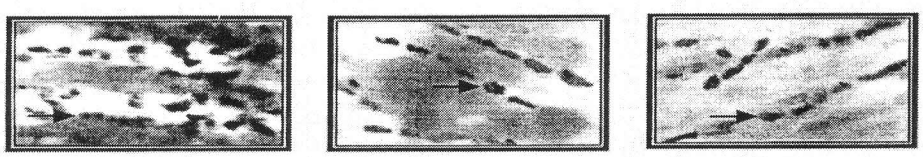
TABLE 3 : Counting results for reg. fiber means in days 14, 21 & 28 of muscle regeneration with the standard deviation , * p value ≤0.001 , # p value ≤0.005 Counting was done at X400 magnification power

ALL FIGURES WERE TAKEN UNDER MAGNIFICATION POWER OF X400



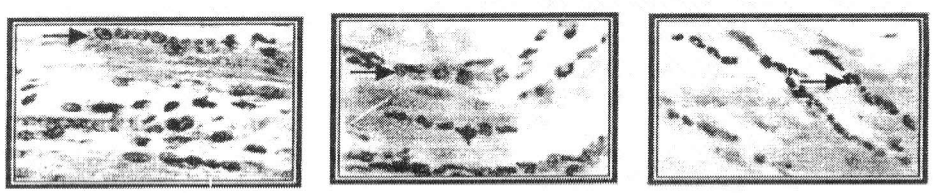
(a) (b) (c)

Fig.1 : Day 3 (Myoblasts) . a ; Control group , H&E stain . b ; Group 4 , MB labelled , NFR stain . c ; Group 4 , imferon labelled , Perl's stain



(a) (b) (c)

Fig. 2 : Day 5 (Myotubes) . a ; Control group , H&E stain . b ; Group 4 , MB labelled , NFR stain . c ; Group 4 , imferon labelled , Perl's stain



(a) (b) (c)

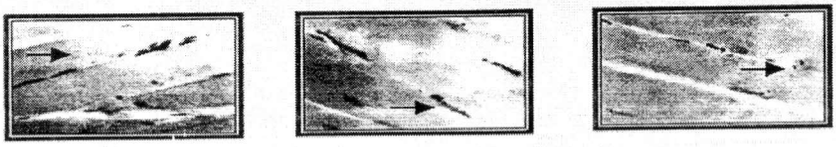
Fig. 3 : Day 7 (Mature myotubes) . a ; Control group , H&E stain . b ; Group 4 , MB labelled , NFR stain . c ; Group 4 , imferon labelled , Perl's stain .

(a) (b) (c)

Fig. 4 : Day 14 (Regenerating fibers) . a ; Control group , H&E stain . b ; Group 4 , MB labelled , NFR stain . c ; Group 4 , imferon labelled , Perl's stain .

(a) (b) (c)

Fig. 5 : Day 21 (Regenerating fibers) . a ; Control group , H&E stain . b ; Group 4 , MB labelled , NFR stain . c ; Group 4 , imferon labelled , Perl's stain .



(a) (b) (c)

Fig. 6 : Day 28 (Regenerating fibers) . a ; Control group , H&E stain . b ; Group 4 , MB labelled , NFR stain . c ; Group 4 , imferon labelled , Perl's stain .

DISCUSSION:

In day 1, the appearance of labeled cells among those infiltrating the injured muscle in the 3rd group with almost normal morphology indicate their persistence and viability in the new tissue. The increase in labeled cells in the 4th group might be due to the initial leucocytosis induced by exercise in this group which give the chance of label uptake for more leucocytes in the same amount of blood used to obtain the buffy coat. MB labeled cells were more than imferon labelled cells possibly due to higher specificity of imferon for monocytes while MB is taken by more cells in the buffy coat. In day 3, the highly significant increase in myoblasts in the 2nd group is possibly due to the effect of exercise which could induce multiple fiber tears with subsequent increase in myoblast supply from the partially injured fibers, or by increasing the affinity of leucocytes to transform into these cells which might explain exercise induced leucocytosis. In the 3rd group, the higher myoblast count compared to control is possibly due to the effect of the implanted buffy coat cells, and this effect could be by transformation of some of them into myoblasts as indicated by the presence of some labeled myoblasts in the area. The higher increase in the 4th group indicates a cumulative and / or synergistic effect between local buffy coat implantation and exercise on the process of muscle regeneration. The effect of dye per se on muscle regeneration is excluded by results of the 5th group which were almost similar to controls (table 1). The increase in myotubes in the 2nd group was the result of increase myoblast count (table 2). The presence of labelled myotubes in

the 3rd and 4th groups with few labeled free myoblasts indicate that some of the implanted buffy coat cells had been transformed into myoblasts and then myotubes. The suggested exercise induced increase in the amount and speed of muscle tissue was elicited by the increase in the mean count of the regenerating muscle fibers in the 2nd and 4th groups with more advanced maturity signs indicated by the wider diameter, less central nuclei, less fiber spacing and less fibrosis as compared to controls. The effect of implanted buffy coat cells on the amount of regenerated tissue was less significant than that of exercise indicated by the weaker rise in the fiber count in he 3rd group, though the enhancing effect of these cells on the speed of regeneration was elicited by the advanced maturity signs in this group compared to controls. The presence of labeled cells inside these new fibers was strong evidence on the participation of some of the implanted buffy coat cells in the process of muscle regeneration. In the 4th group, the highest fiber count associated with best maturity signs compared to all groups indicated the synergistic and / or cumulative effect of exercise and local buffy coat implantation on the process of muscle regeneration (table 3). The presence of regenerated fibers labeled with MB and imferon indicates that some buffy coat cells had transformed into muscle forming cells, and the relatively high imferon labeled fibers with its relative monocyte specificity indicate that most of the transformed cells were monocytes.

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