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RESEARCH ARTICLE

Histopathological changes in extraocular muscles of rabbits following injection of bupivacaine 5mg/Ml versus 7.5mg/Ml

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ABSTRACT

Purpose: To compare the histopathological effects of injecting two concentrations of Bupivacaine (5 mg/ml and 7.5 mg/ml) in the superior rectus muscle of rabbits, and to compare these to conventional extraocular muscle surgery in previous studies.

Methods: Eighteen albino rabbits' eyes were used. The superior rectus muscles were injected with Bupivacaine 5 mg/ml (Group B5, 10 eyes) or 7.5 mg/ml (Group B7, 8 eyes). The rabbits were sacrificed and eyes enucleated 6 weeks later for histopathological evaluation. Results were compared to the average of those obtained, by three previous studies, after conventional superior rectus resection in rabbits.

Results: Foreign body reaction was absent in all specimens. Conjunctival and scleral inflammation, perimuscular adhesions, intramuscular fibrosis, conjunctival and scleral oedema and muscle atrophy were higher in group B7, while conjunctival hyperaemia and muscle hypertrophy were higher in group B5 ($p > 0.05$). On comparison to conventional surgery, conjunctival inflammation and hyperaemia, foreign body reaction, and adhesions were less after bupivacaine injection ($p > 0.05$ for all except for intensity of conjunctival inflammation in B5 versus conventional surgery). Scleral inflammation was more frequent after bupivacaine injection ($p < 0.05$). Muscle fibrosis was more frequent in group B7 and conventional surgery than in group B5 ($p > 0.05$).

Conclusions: Both Bupivacaine concentrations effectively produced the desired muscle hypertrophy and fibrosis, so the lower concentration may be used for muscle strengthening to correct strabismus. Bupivacaine injection, although produced no foreign body reaction, did not significantly lower the development of undesired postoperative adhesions and caused more scleral inflammation.

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Bupivacaine; fibrosis; adhesions; extraocular muscles; hypertrophy; atrophy

Introduction

Local anaesthetic agents used in ocular surgery, especially bupivacaine, can be associated with postoperative strabismus. The latter usually presents with initial muscle paresis, followed by muscle overaction, stemming from late-phase fibrosis and possibly muscle hypertrophy[1]. Strabismus inadvertently occurring after bupivacaine (BUP) injection as a local retrobulbar anaesthetic agent for cataract surgery[2,3], triggered the use of this agent to treat strabismus[4].

A previous study determined the changes in the cross-sectional area (CSA) of myofibers and their subtype distribution based on the myosin isoform expression after 1.5% BUP injection in the EOM of rabbits. Myofiber area measurement decreased 7 days after BUP injection followed by an increasing trend after 28 days and normalisation after 92 days. The proportion of slow myosin-positive fibres increased 60 days after injection, but there was no statistically significant difference in fast myosin-positive fibres. No increase of endomysial fibrous tissue was observed[5].

A subsequent study compared the cross-sectional area of fibres positive for myosin type 1 in the global and orbital layers after BUP 1.5% injection in the superior rectus of rabbits, and documented an increase in the CSA of myosin 1 type fibres in the global, but not in the orbital layer, 60 days post-injection[6].

The aim of this study was to compare the histopathological effects of injecting two different concentrations of BUP (5 mg/ml and 7.5 mg/ml) in the superior rectus muscle of rabbits, and compare these to the histopathological effects of conventional surgery as documented by previous studies. Particular attention was paid to the evaluation of fibrosis and adhesions as possible complications.

Methods and materials

The study was conducted in the Ophthalmic and Pathology departments of Cairo University Hospitals. Ethical committee approval was obtained (approval number CUIIIF321).

Eighteen eyes of albino rabbits, weighing approximately 2 kg, were used. The rabbits were anaesthetized with intramuscular Xylazine hydrochloride (5 mg/kg) and Ketamine hydrochloride (3.5 mg/kg), with additional doses as required.

The superior rectus muscles of both eyes of each rabbit were included in the study. Muscles were assigned to either injection with BUP 5 mg/ml (Group B5, 10 eyes) or 7.5 mg/ml

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(Group B7, 8 eyes). After depressing the eye globe with a strabismus hook applied in the lower fornix, the superior rectus muscle was grasped with forceps and 1 ml was injected, using an insulin syringe, through intact conjunctiva.

Six weeks later, after muscle healing, the rabbits were sacrificed. A limbal suture was placed corresponding to the plane of the superior rectus muscle to aid in its identification. Eyes were enucleated and fixed in 10% formalin solution. The enucleation specimen included an intact block of superior rectus muscle, together with underlying and surrounding sclera, as well as overlying and surrounding Tenon's capsule and conjunctiva.

A single pathologist, blinded to the procedure, examined all specimens. Serial cuts were made through the superior rectus muscle, sclera, Tenon's capsule and conjunctiva. The formalin-fixed sections were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. The paraffin embedded blocks were serially sectioned at 5-micron thickness. The sections were stained with haematoxylin-oeosin stain to be examined and graded microscopically for the presence of conjunctival, scleral and foreign body (F.B.) inflammation, conjunctival vascularity, amount of fibrous tissue formation (adhesions) between rectus muscle and sclera, Tenon's capsule and conjunctiva, rectus muscle fibrosis, muscle atrophy, muscle hypertrophy and subepithelial conjunctival and scleral oedema. The histopathologically evaluated and graded parameters are shown in Table 1. A semi-quantitative method was used for scoring.

Results of the current study were compared to conventional superior rectus resection in rabbits, performed in the control groups of 3 previous studies authored by some of the authors of the present study[6,9,10] that utilized the same scoring system as the present one. The average of the scores obtained by the three studies was used for statistical comparisons.

Data were statistically described in terms of mean ± standard deviation (±SD), median and range, or frequencies (number of cases) and percentages where appropriate. Numerical data were tested for the normal assumption using Shapiro–Wilk test. Comparison of numerical variables between the study groups was done using Kruskal–Wallis test with Mann–Whitney U-test as *post hoc* multiple 2-group comparisons after applying Bonferroni adjustment for multiple comparisons. For comparing categorical data, Chi-square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5. Two-sided p values less than 0.05 were considered statistically significant. All statistical calculations were done using the computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

Table 1. Parameters for histopathologic evaluation (7, 8, with modification).

Parameters	Values
Conjunctival inflammation	0 = no inflammation 1 = a few lymphocytes and plasma cells beneath epithelium 2 = mild inflammatory infiltrate composed of lymphocytes, plasma cells and polymorphnuclear leucocytes beneath epithelium and congestion 3 = grade two plus neutrophils in the epithelium 4 = high concentrations (collections) of lymphocytes, plasma cells, polymorphnuclear leucocytes and histiocytes (both intraepithelial and subepithelial) and ulceration
Scleral inflammation	0 = absent 1 = present
Foreign body reaction	0 = absent 1 = present
Conjunctival vascularity	0 = normal vascularity 1 = mildly increased vascularity suggestive of ongoing inflammation 2 = moderately increased vascularity 3= severely increased vascularity
Adhesion between rectus muscle and surrounding tissues (sclera, Tenon's capsule, and conjunctiva)	0 = no fibrosis 1 = mild perimuscular fibrotic reaction (stained collagen is detectable only in thin bands immediately adjacent to muscle) 2 = easily detected thick bands 3 = well-developed, dense bands of collagen 4 = a severe fibrotic response replacing large areas
Rectus muscle fibrosis	0 = absent 1 = present
Muscle atrophy	0 = absent 1= mild 2= marked
Muscle hypertrophy	0 = absent 1 = present
Subepithelial conjunctival and scleral oedema	0 = absent 1 = present

Results

Results of histopathological evaluation of the 18 rabbit eyes are shown in Table 2. These included 10 eyes in group B5 and 8 in group B7. Foreign body reaction was absent in all specimens. Conjunctival and scleral inflammation (Figure 1), perimuscular fibrosis and adhesions, intramuscular fibrosis, conjunctival and scleral oedema (Figure 2) and muscle atrophy (Figure 3) were insignificantly higher in group B7, while conjunctival hyperaemia (Figure 4) and muscle hypertrophy were insignificantly higher (Figure 5) in group B5 ($p > 0.05$).

Table 2 also shows the average scores obtained after conventional superior rectus resection in three previous studies[6,9,10], together with statistical comparisons to the current one. The severity and frequency of conjunctival inflammation and hyperaemia as well as adhesions were lower after bupivacaine injection than conventional surgery. All were statistically insignificant except for the intensity of conjunctival inflammation in B5 versus conventional surgery. The frequency of scleral inflammation was prominently and significantly higher after bupivacaine injection than conventional surgery. Foreign body reaction was absent after bupivacaine injection but present after conventional surgery. Muscle fibrosis was more frequent in group B7 and conventional surgery than in group B5 but of statistical insignificance.

Discussion

Bupivacaine injection results in myotoxicity and necrosis of extraocular muscles, except in the basal lamina, nerves, and satellite cells (stem cell of myofibrils) (1). With the release of growth factors from the damaged muscle fibres, satellite cells

Table 2. Statistical analysis of the scores of the different parameters according to histopathological examination.

	Group[a]	Conjunctival inflammation[b]	Scleral inflammation[c]	F.B. reaction[c]	Conjunctival hyperemia[b]	Perimuscular fibrosis and adhesions[b]	Intramuscular fibrosis[c]	Conjunctival and scleral oedema[c]	Muscle Atrophy[b]	Muscle Hypertrophy[c]
Mean+/-SD	B5 (n = 10)	1.1+/-1.197	–	–	0.7+/-0.675	1.5+/-1.179	–	–	0.6+/-0.699	–
	B7 (n = 8)	1.38+/-1.408	–	–	0.38+/-0.518	1.75+/-1.282	–	–	1+/-0.756	–
	Conventional surgery (n = 34/3 = 11)[d]	2.34+/-0.88	–	–	0.97+/-0.76	2.195+/-0.92	–	–	–	–
P values	B5 vs B7[e]	.640	–	–	.294	.677	–	–	.248	–
	B5 vs Conventional	.014	–	–	.403	.147	–	–	–	–
	B7 vs Conventional	.085	–	–	.076	.390	–	–	–	–
Median	B5	1(0-3)	–	–	1(0-2)	1(0-3)	–	–	0.5(0-2)	–
(minimum -maximum)	B7	1(0-4)	–	–	0(0-1)	1.5(0-4)	–	–	1(0-2)	–
Frequency of	B5	60	60	0	60	80	50	10	50	60
occurrence (%)	B7	75	62.5	0	37.5	87.5	75	12.5	75	37.5
	Conventional surgery (n = 34/3 = 11)[d]	100	9	45.5	73	100	73	–	–	–
P values	B5 vs B7[e]	.638	.627	–	.637	1.000	.367	1.000	.367	.637
	B5 vs Conventional	.076	.045	.054	.877	.415	.534	–	–	–
	B7 vs Conventional	.319	.048	.090	.287	.870	.677	–	–	–

[a]Eye name denotes group (B5 = Bupivacaine 5 mg/ml group, B7 = Bupivacaine 7.5 mg/ml group) followed by rabbit number.
[b]Numerical variables.
[c]Categorical variables.
[d]The average of the results obtained in the control group (undergoing superior rectus resection) of 3 previous studies, utilising the same scoring system as the present study, was used (9–11).
[e]*p* values were calculated using Mann-Whitney test for numerical variables, and Fisher's Exact test for categorical variables.
SD: standard deviation.

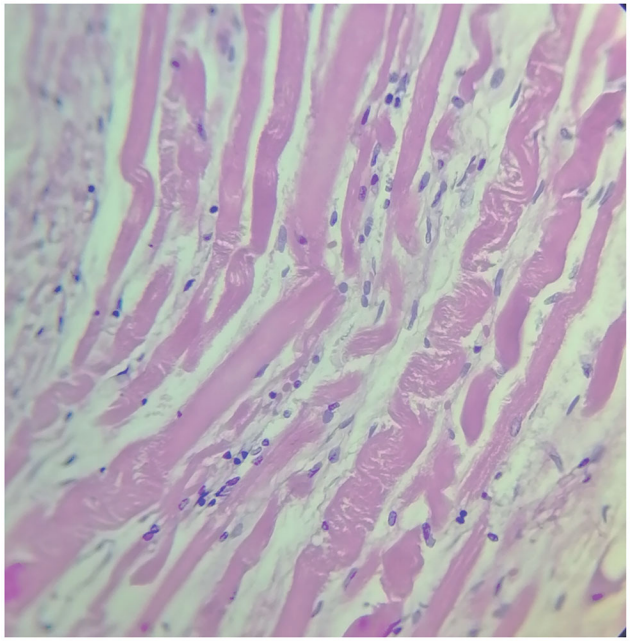

Figure 1. Subepithelial dense lymphoplasmacytic inflammatory cellular infiltrates entangling focal lymphoid aggregates (Haematoxylin–eosin stain; original magnification × 400).

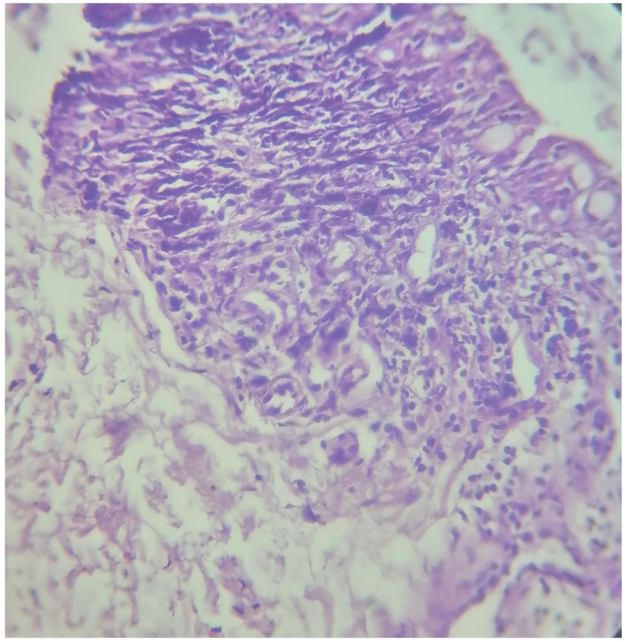

Figure 2. Marked stromal edema of subconjuctival epithelium (Haematoxylin-oeosin; original magnification × 400).

are activated to proliferate and form new muscle fibres[12]. This regeneration process results in hypertrophy of the affected muscle[13]. Scott et al. (2009) treated esotropic patients with a high-concentration bupivacaine injection into the lateral rectus muscle (0.75% and greater), thus taking advantage of the induced muscle overaction[14].

The present study compared the 6-week postoperative effect of bupivacaine 7.5 mg/ml to 5 mg/ml, to determine if a lower concentration than 7.5 mg/ml can still be effective to produce muscle hypertrophy. Muscle hypertrophy was detected in 60% of group B5 specimens, while it was present in only 37.5% of group B7, which entails more frequent

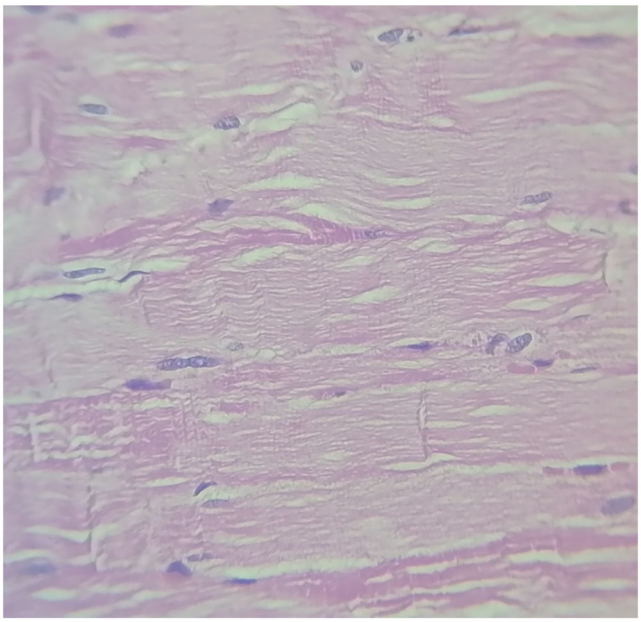

Figure 3. Atrophic thinned out muscle fibres with pyknotic nuclei (Haematoxylin-oeosin stain; original magnification × 400).

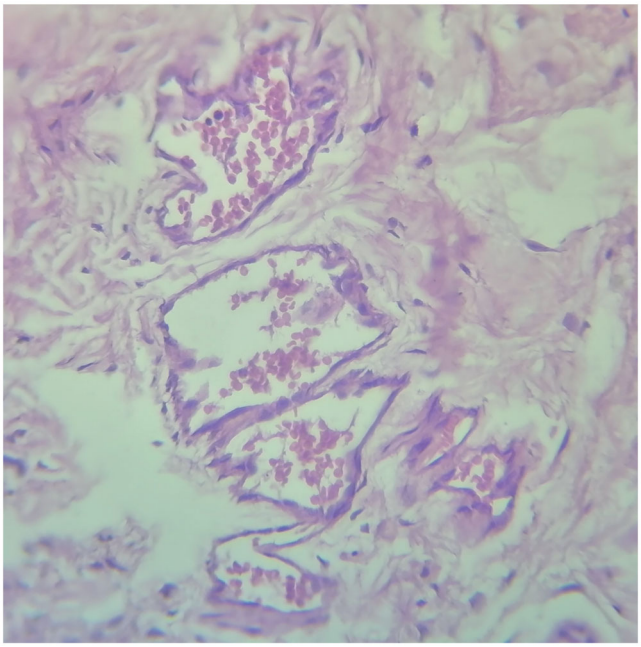

Figure 4. A focus showing closely packed congested vascular spaces (Haematoxylin-oeosin stain; original magnification × 400).

hypertrophy with the 5 mg/ml concentration. The difference was statistically insignificant, so could merely be related to chance. Nevertheless, it still showed that the 5 mg/ml concentration is still effective in producing muscle hypertrophy. Intramuscular fibrosis was detected in 50% of B5 specimens and 75% of B7 specimens. Although the incidence of fibrosis was lower with the 5 mg/ml concentration, yet this was not statistically significant. Muscle hypertrophy and fibrosis could, therefore be obtained by the 5 mg/ml as well as the 7.5 mg/ml concentrations of bupivacaine.

A previous histopathological study was conducted to compare the effects of injecting different bupivacaine concentrations in rabbits' extraocular muscles (0.75, 0.38 and 0.19%).

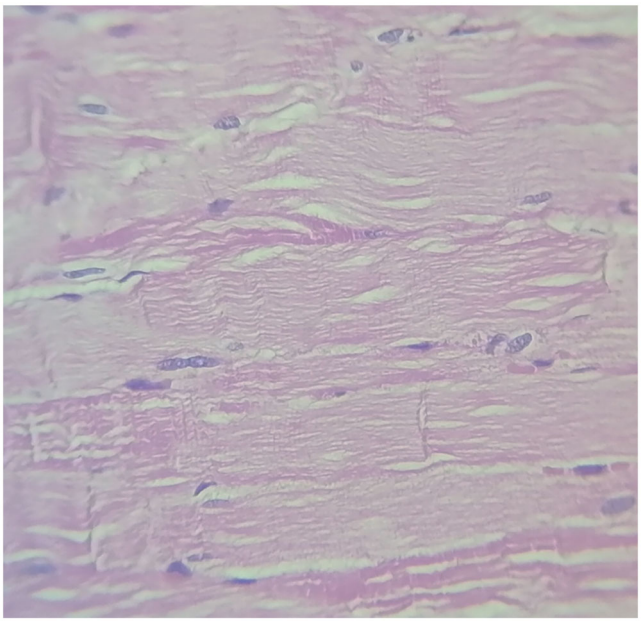

Figure 5. Compensatory hypertrophy of the included muscle fibres with thickened calibre (Haematoxylin-oeosin stain; original magnification × 400).

At 1 month, only 0.75% bupivacaine-injected muscles displayed areas of regenerated muscle fibre cells with foci of scar formation in 50% of specimens. There was no visible scar formation in muscles injected with any bupivacaine concentration lower than 0.75%[1]. This study, however, compared the 0.75% concentration to ones as low as 0.38 and 0.19%, while the present study compared the former to a higher concentration (5 mg/ml) than the latter study.

Later, two further studies were conducted by Hopker and co-authors[5,6] to analyse the histopathological effects of BUP 1.5% injection in rabbits' extraocular muscles. These two studies used a higher concentration than the present and previous studies. Moreover, these two studies performed a staged evaluation at 7, 28, 60 and 92 days, while the present study performed it at 6 weeks only. Although the present studies evaluated multiple parameters (Tables 1 and 2), these previous ones determined only the changes in the cross-sectional area (CSA) of myofibres and their subtype distribution based on the myosin isoform expression. Myofiber area measurement decreased 7 days after BUP injection followed by an increasing trend after 28 days and normalisation after 92 days. The present study evaluated muscle hypertrophy at 6 weeks. Atrophy was mild in both groups and hypertrophy was documented in 60% of Group B5 and 37.5% of Group B7. Comparison to the 2 previous studies is impossible due to different timing and parameters used for evaluation.

One of the major problems in strabismus surgery is the development of postoperative adhesions. These adhesions, which may involve the conjunctiva, Tenon's capsule, orbital fat, sclera and extraocular muscles, may result in motility problems affecting the surgical outcome.[7] The present study compared the frequency and severity of adhesions after two concentrations of bupivacaine injection. These were insignificantly and slightly lower with Bupivacaine 5 mg/ml. The amount of adhesions developing after bupivacaine injection was also compared to that occurring after conventional

strabismus surgery in three previous studies[6,9,10]. Adhesions occurred in 100% of specimens of the previous studies as compared to 80% of group B5 ($p = 0.415$) and 87.5% of group B7 ($p = 0.870$). The mean adhesions score was 2.1 9 5 ± 0.92, on average, in the previous studies, which was insignificantly higher than the 1.5 ± 1.179 and the 1.75 ± 1.282 scores obtained in groups B5 ($p = 0.147$) and B7 ($p = 0.390$), respectively. Accordingly, Bupivacaine had no privilege in significantly diminishing the development of adhesions, after extraocular muscles surgery, despite the absence of any surgical dissections. These adhesions might be caused by a reaction to drug itself. Similarly, the prominently higher frequency of scleral inflammation after Bupivacaine injection (60% and 62.5% in groups B5 and B7, respectively) versus conventional surgery (9%), entails a possible reaction to the drug. On the other hand, the absence of foreign body reaction after Bupivacaine injection and its presence after conventional surgery is probably related to the suture material used only in the latter.

Limitations of the present study include the following. First, the study lacked a control group. A control group, where saline is injected in extraocular muscles is recommended in future studies. Second, immunohistochemistry with anti-CD45, CD3 and CD20, desmin and alpha-smooth muscle actin antibodies as well as special staining such as Masson-Fontana, looking at inflammatory cell infiltration, muscle atrophy, and fibrosis, respectively, is lacking in the present study and advised in future studies. Third, Also, imaging software would have been preferred to quantify the morphological alterations including inflammatory cell infiltrates and fibrosis.

In conclusion, both concentrations of Bupivacaine (5 mg/ml and 7.5 mg/ml) were effective in producing the desired muscle hypertrophy and fibrosis, so the lower concentration may be used for the purpose of producing a muscle-strengthening effect to correct strabismus. Bupivacaine injection, however, did not have the privilege of significantly minimising postoperative perimuscular adhesions. Moreover, a risk of scleral inflammation was significantly higher after Bupivacaine injection than conventional surgery.

Disclosure statement

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