In vitro evaluation of the effects of 5-Fluorouracil and Mitomycin-C on canine subconjunctival and subtenon's fibroblasts, with subsequent in vivo clinical trial in combination with the Baerveldt drainage device

by

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1. GENERAL INTRODUCTION

Thesis Organization

The following thesis represents a compilation of two separate manuscripts. The first chapter provides a referenced overview of the current state of knowledge of glaucoma management in the dog. The second chapter details results from the initial *in vitro* clinical trial and their interpretation. Chapter 3 continues the experiment by investigating the clinical effects in the *in vivo* environment. Chapter 4 summarizes the general conclusions which were drawn from information obtained in both the *in vitro* and *in vivo* clinical trials.

Literature Review

Glaucoma represents one of the leading causes of blindness, both in the dog[1] and man. Although this disease process has been recognized for many years, satisfactory medical and surgical control continues to elude both the veterinary and human ophthalmologist. It is generally agreed upon by most veterinary ophthalmologists that control of this disease process cannot be achieved through medical management alone. A variety of historical surgical techniques have been described. Included are iridencleisis, cyclodialysis, peripheral iridectomy, combined iridencleisis-cyclodialysis, corneoscleral trephination-peripheral iridectomy, cyclodialysis-iridocyclectomy, and posterior sclerectomy-cyclodialysis-transscleral iridencleisis.[1,2] Although routinely employed in man, these techniques have fallen out of use in domestic animals. This dichotomy in clinical success appears directly related to the increased postoperative inflammatory response observed in most of our domestic animal species.

One of the new and evolving areas of glaucoma research is the use of anterior chamber drainage devices. A wide variety of implants are currently being used for glaucoma management in man[3-10] and the dog.[11-14] All incorporate a hollow silicon tube which functions as a sclerostomy opening, shunting aqueous humor to the adjoining polypropylene or silicon foot plate (scleral explant). Emergent fluid collects in the surrounding filtering bleb and is taken up by the regional vascular channels.[12] Following implantation, the developing fibrosis at the site of the filtration bleb acts as the main regulator of intraocular pressure (IOP).

Although none of the currently available models have proven to be superior under all conditions, initial studies suggest this to be an improved technique for the control of canine glaucomas.[11,12] In man, synthetic drainage devices are restricted for use in those individuals who are refractory to all other medical and surgical efforts.[9] Despite this skewing of potential candidates, surgical outcome is also generally rewarding.

The most significant complication of glaucoma filtration or drainage device (seton) surgery, in human or canine patients, is excessive fibrosis at the site with resultant premature failure of the procedure.[15] This appears to result from the proliferation of subconjunctival fibroblasts with concurrent synthesis of collagen and other extracellular material, all of which are part of the normal wound healing process.[16] Numerous antimetabolites have demonstrated the ability to suppress this scarring process through a presumed direct effect on the regional fibroblasts. Within this group, 5-fluorouracil (5FU) and mitomycin C (MMC) represent the two most commonly used agents in human ophthalmology. While numerous human clinical trials support the beneficial effects of concurrent antimetabolite therapy, only a limited number of reports have attempted to quantify their effects using cell culture techniques.[17-21] Similar reports have not been identified in the veterinary literature.

The purpose of this project was two-fold. The initial goal was to obtain preliminary information regarding the effects of 5FU and MMC on cultured canine fibroblast growth and proliferation. Since fibroblast migration is intimately involved with the process of fibrosis, we also investigated the effects of 5FU and MMC on cell motility using an agarose droplet motility assay. The second goal of the study was to extrapolate findings from the *in vitro* portion of the study by investigating the effects of single dose MMC therapy on normotensive globes undergoing glaucoma seton surgery. It was hoped that *in vivo* clinical studies would further support continued fibroblastic inhibition with resultant prolongation of filtration bleb viability and regulation of IOP. The authors were unable to identify similar clinical trials in the human or veterinary literature.

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2. *IN VITRO* EVALUATION OF THE EFFECTS OF 5-FLUOROURACIL AND MITOMYCIN-C ON CANINE SUBCONJUNCTIVAL AND SUBTENON'S FIBROBLASTS

A paper to be submitted to Veterinary & Comparative Ophthalmology

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Abstract

The authors investigated the effects of mitomycin C (MMC) and 5 fluorouracil (5FU) on proliferation and motility of canine subconjunctival fibroblasts. A dose and time dependent inhibition of fibroblast growth was observed following 5, 10, 15, and 20 days of treatment. The minimum concentration of MMC required to cause a significant ($p<0.05$) reduction in cell numbers was 1/10th that required for 5FU for all durations of exposure. Inhibitory effects were reversible for both MMC and 5FU at 1×10^{-2} mg/l following up to 4 days of treatment. Beyond this interval, results did not differ significantly from continuous treatment cells. Use of increased antimetabolite concentration (5FU 1×10^{-1} mg/l) further reduced the reversibility period with growth inhibition reaching a plateau following 2 days of exposure. Use of fluorescein labeled anti-actin antibody confirmed structural alterations of the polymerized F-actin in 30% of cells when treated with MMC (1×10^{-3} mg/l) or 5FU (1×10^{-2} mg/l). Neither drug caused a significant ($p<0.05$) alteration in fibroblast migration when compared to non-treated control cells. Results obtained from this study suggest that the inhibitory effects of MMC and 5FU on cultured canine fibroblasts act primarily through inhibition of cellular proliferation. Their influence on cellular adhesion and migration remain unresolved as conflicting results were obtained from F-actin and agarose motility studies. Interpretation of results from dose-inhibition and drug reversibility studies support the use of high-dose single treatment protocols for subsequent *in vivo* fibroblast inhibitory efforts.

Introduction

Glaucoma filtration surgeries are routinely performed in those human patients who respond poorly to conventional medical therapy. However, as was observed in canine glaucoma patients, fibrosis at the site of the

surgically created filtration bleb has resulted in premature failure of these procedures in several patients.[1] This appears to result from the proliferation of subconjunctival fibroblasts with concurrent synthesis of collagen and other extracellular material, all of which are part of the normal wound healing process.[2]

Numerous antimetabolites have demonstrated the ability to suppress this scarring process through a presumed direct effect on the regional fibroblasts. Within this group, 5-fluorouracil (5FU) and mitomycin C (MMC) represent the two most commonly used agents in human ophthalmology. 5FU has been shown to interfere with the S and G2 phases of the cell cycle resulting in impaired DNA and RNA biosynthesis, respectively.[3] This specificity targets the drug towards actively proliferating cells. Perhaps the most widely accepted treatment protocol involves the postoperative injection of 5 mg of 5FU into the subconjunctival space twice daily for 7 days followed by daily doses for the ensuing week.[4] Although numerous, often self-limiting, complications such as conjunctival wound leaks and corneal defects have been reported,[4] more serious problems such as recalcitrant corneal ulceration, opacification and perforation have also been observed.[5] These observations, combined with the inconvenience of numerous postoperative visits, has prompted investigation into alternative antimetabolite agents.

MC is an antitumor antibiotic isolated from *Streptomyces caespitosus*. Its antitumor effect is attributed to the inhibition of DNA replication leading to cell death.[6] MMC alkylates and crosslinks DNA and, in addition, may generate superoxide and hydroxyl radicals. It has also been shown to inhibit synthesis of DNA, RNA and protein.[7] The combination of these features are believed responsible for the clinically observed long-term inhibitory effects on cellular proliferation.[6,8-11] Unlike the 5FU treatment protocol, MMC is typically administered only once during the perioperative period.[10]

While numerous human clinical trials support the beneficial effects of concurrent antimetabolite therapy, only a limited number of reports have attempted to quantify their effects using cell culture techniques.[12-16] Similar reports were not identified in the veterinary literature. The purpose of this project was to obtain preliminary information regarding the effects of 5FU and MMC on cultured canine fibroblast growth

and proliferation. Since fibroblast migration is intimately involved with the process of fibrosis, we also investigated the effects of 5FU and MMC on cell motility using an agarose droplet motility assay. Fluorescein labeled anti-actin antibody staining was used to visually compare polymerized cytoplasmic F-actin filaments of control and antimetabolite treatment groups. F-actin filaments are believed to represent the major cytoskeletal protein responsible for cell adhesion and migration.[16]

Materials and Methods

Fibroblast Cultures

Subconjunctival tissues were obtained from four dogs concurrently used for non-ocular student surgical exercises. Removal of the dorsal bulbar conjunctiva enabled collection of a 20 mm x 20 mm calotte of exposed subconjuctival tissues including Tenon's capsule. Sharp dissection was used to divide the sample into multiple 2 mm x 2 mm blocks. Tissues were then placed in 40 ml (75 sq.cm.) tissue culture flasks (Costar Plastics, Rochester, NY) with minimal essential media (MEM) (Gibco Biologics, Grand Island, NY) fortified with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO), gentamicin sulfate, 25 mg/500 ml MEM, (Gentocin,® Schering-Plough Animal Health, Kenilworth, NJ) and Amphotericin B, 1 mg/500 ml MEM, (Fungizone,® ER Squibb & Sons Inc., Princeton, NJ). An attempt was made to orient the sample such that Tenon's capsule was in direct contact with the media. Cells were incubated at 37°C in 5% CO_2 (Heinicke Co., Portland, OR) and allowed to reach confluence before being passaged. Media was replaced every 48 to 72 hours in all cell cultures. At the time of passage, media was decanted, cells were treated with a 0.05% trypsinizing solution (Gibco Biologics, Grand Island, NY) and incubated for 5 minutes. The flask was gently agitated and the resultant suspension of cells was pipetted ("Pipet-aid", Drummond Scientific Co., Broomall, PA) to recipient flasks or tissue culture plates. A 0.25 ml aliquot was stained using 0.4% trypan blue (Sigma Chemical Co., St. Louis, MO) and cell concentrations were manually determined using an Improved Neubaur

Hemocytometer (Fisher Scientific, Pittsburgh, PA) and inverted microscopy (Leitz Diavert, Henry Louis Inc., Iowa City, IA). Cells were passaged three to eight times. All passages were performed under a Steril GARD Hood (The Baker Comp. Inc., Sandford, ME).

Dose-Inhibition Studies Of Cell Proliferation

One milliliter (1.8×10^4 cells/ml) of a fibroblast suspension was pipetted into each well of twelve 24-well tissue culture plates (Costar Plastics, Rochester, NY). Cells were incubated for 24 hours at 37°C and 5% CO_2 to allow attachment to the substratum. The initial medium was replaced with fresh MEM containing the following concentrations of antimetabolites: 5FU from 1.0×10^{-4} mg/l to 10 mg/l (Aldrich Chemicals, Milwaukee, WI) or MMC from 1.0×10^{-4} mg/l to 1.0 mg/l (Aldrich Chemicals, Milwaukee, WI). Drug-free media were used in control wells. Three plates were randomly assigned to one of four groups and followed for 5, 10, 15, or 20 days of treatment. Wells were monitored daily using inverted microscopy and photographed weekly with phase contrast techniques. At the termination of each study period the cells of each well were trypsinized and counted as previously described under "Fibroblast Culture". Six replicates were performed for each drug concentration and treatment duration investigated. Percent-growth of the fibroblasts relative to the control was defined as (B/A) x 100, where A = average number of cells in drug-free control wells and B = actual number of cells in a drug-treated well, as per Yamamoto et al.[16] Results were statistically analyzed using the Student's *t* test.

Reversibility Of Drug-Induced Growth Inhibition

One milliliter (1.8×10^4 cells/ml) of a fibroblast suspension was pipetted into each well of six 24-well tissue culture plates. Cells were incubated for 24 hours at 37°C and 5% CO_2 to allow attachment to the substratum. Wells were then randomly assigned to one of the following groups: control group, continuous treatment group, or one of three washout groups where media containing a selected antimetabolite were removed following 2, 4, or 7 days of treatment and replaced with the drug-free media. Only drug-free media

were used in the control group. In the continuous treatment group the cells were treated for the duration of the study with the assigned antimetabolite without drug washout. Antimetabolite concentrations used included 5FU at 1 x 10^{-2} mg/l or 1 x 10^{-1} mg/l and MMC at 1 x 10^{-3} mg/l or 1 x 10^{-2} mg/l. Cells were examined daily for 14 days using inverted microscopy, and on the last day photographed, typsinized and counted. Percent-growth of the fibroblasts relative to the control group was determined. Four replicates were performed for each group examined. Results were statistically analyzed using the Student's *t* test.

Localization Of F-Actin

A 12 mm diameter circular glass coverslip (Fisher Scientific, Pittsburgh, PA) was placed within each well of two 24-well tissue culture plates. To this was added 1 ml (1.55 x 10^4 cells/ml) of a suspension of fibroblasts. Cells were incubated for 24 hours at 37°C and 5% CO_2 to allow attachment to the coverslips. Wells were then randomly assigned to one of three groups: control, 5FU (1 x 10^{-1} mg/l to 1 x 10^{-3} mg/l) or MMC (1 x10^{-1} mg/l to 1 x 10^{-3} mg/l). As in earlier experiments, control wells received drug-free media. On days 1, 3, 5, 7, 9 and 11 one coverslip was removed from each group. Cells were fixed to the glass coverslips using a 10 minute treatment of a 30% methanol : 70% acetone solution. Following subsequent treatment with an affinity-isolated anti-actin antibody preparation (Sigma Chemical Co., St. Louis, MO), the cells were incubated for 45 minutes at 37°C with 5% CO_2, rinsed in distilled water and treated with an affinity purified antibody (Fluorescein labeled Goat anti- Rabbit IgG) (Kirkegaard & Perry Labs. Inc., Gaithersburg, MD). After an additional 45 minute incubation period, the coverslips were rinsed with distilled water, air dried and mounted to glass slides for examination and photography using fluorescent microscopy (Leitz Wetzlar Orthoplan, Henry Louis Inc., Iowa City, IA).

Agarose Droplet Motility Assay

Fibroblasts were trypsinized, counted and centrifuged (3,000 RPM) (Beckman® TJ-6, Palo Alto, CA) to obtain a pellet of 3.5×10^6 cells. Cells were then covered with 0.1 ml of warmed 0.3% agarose (SeaKem® ME Agarose, FMC Bio Products, Rockland, ME) dissolved in serum-free MEM and agitated to form a uniform cellular suspension. Agarose cell droplets of a 5-µl volume were pipetted into the center of each well of a 96-well tissue culture plate (Costar Plastics, Rochester, NY). The plate was then refrigerated for 5 minutes at 4°C to promote gelatinization of the agarose. After random assignment to a particular treatment group, the pellets were covered with serum-containing MEM supplemented with 1.0×10^{-4} mg/l to 1 mg/l of MMC or 1.0×10^{-2} mg/l to 10 mg/l of 5FU. Drug-free media were used in control wells and one group of serum-free MEM. Horizontal and vertical dimensions of each pellet (Days 0 - 7) were used to calculate mean surface areas with values expressed as a ratio to the serum-containing control group. Results were statistically analyzed using the Student's *t* test. Eight replicates were performed for each treatment group.

Results

Dose-Inhibition Studies Of Cell Proliferation

Figures 1, 2, 3, and 4 demonstrate a dose-dependent inhibition of fibroblast growth following exposure to either 5FU or MMC over 5, 10, 15, or 20 days of treatment. Examination of all four figures yielded a trend towards improved fibroblast growth as 5FU concentration was increased from 1 mg/l to 10 mg/l. This was most evident in the 15 and 20 day studies. Regression analysis using cubic or quadratic equations derived from original data failed to support drug concentration as the causative factor for these rises (r-square = 0.05); thereby suggesting an additional, unidentified, variable (r-square = 0.95). The Student *t* test was used to determine the minimum antimetabolite concentration necessary to obtain a statistically significant ($p < 0.05$)

reduction in fibroblast growth relative to control wells. Figure 5 demonstrates that not only was this minimum value reduced with increasing duration of exposure to the selected antimetabolite, but also that at any given duration of treatment, the minimum required concentration of MMC was 1/10th that of 5FU.

Gross cytologic changes were monitored through the use of phase contrast microscopy. Figure 6 demonstrates the typically homogenous arrangement of spindle-shaped fibroblasts observed when grown to confluence in drug-free control wells. Figures 7, 8, and 9 demonstrate signs of advancing cytotoxicity as evidenced by increased granularity, decreased cell to cell adhesion, and ultimately rounding of cells following loss of their cytoplasmic processes. Rounding of cells was generally associated with loss of adhesion to the substratum with subsequent cell disappearance from the monolayer.

Reversibility Of Drug-Induced Growth Inhibition

Results of this study are depicted in figure 10. All continuous treatment groups, excluding MMC 1 x 10^{-3} mg/l, yielded a statistically significant ($p< 0.05$) reduction in fibroblast growth relative to control wells. No statistical reduction was observed with MMC at 1 x 10^{-3} mg/l when washed out after 2, 4, or 7 days of exposure. All other washout results, excepting 5FU at 1 x 10^{-2} mg/l (2 days) showed significant ($p< 0.05$) reduction in growth relative to control wells.

Washout wells were then compared against antimetabolite matched continuous treatment groups (fig. 11) in order to determine at what time the washout groups failed to significantly differ ($p < 0.05$) from the continuous treatment group. Cells exposed to 2, 4 or 7 days of MMC at 1 x 10^{-3} mg/l did not differ significantly with cells from continuous treatment (fig. 11) or drug-free control wells (fig. 10). No significant ($p < 0.05$) difference was observed between continuous treatment and washout groups using 5FU 1 x10^{-1} mg/l. Although all values were statistically reduced from control values (fig. 10), near maximum inhibition had already been achieved following 2 days of treatment (fig. 11). When analyzing MMC and 5FU at 1 x 10^{-2} mg/l, a significant difference ($p < 0.05$) was observed between continuous treatment and washout groups until

day 4. Failure to recognize a similar difference at day 7 suggested that, at least for this concentration of the antimetabolites, the inhibitory effect was maximized between 4 and 7 days of therapy.

Localization Of F-Actin

At the onset of this experiment all cells demonstrated the normal arrangement of F-actin, with stress fibers observed to extend into all cellular processes including lamellipodia, filopodia and ruffled membranes (fig. 12). Conversely, approximately 30% of cells treated with either MMC at 1×10^{-3} mg/l or 5FU at 1×10^{-2} mg/l were found to have normal cytoplasmic architecture with an intermittent fluorescent staining pattern to their actin filaments (fig. 13). These findings were most apparent at days 3 and 5 with some evidence of cell loss by day 11. Additionally, all viable cells regardless of treatment protocol, demonstrated a perinuclear accumulation of fluorescein. Regional protein synthesis, including the formation of monomeric actin, was presumed responsible for these observations (figs. 12, & 13). Even in cells where gross detachment and rounding up were observed, F-actin distribution, albeit intermittent, extended throughout the entire cytoplasm.

Agarose Droplet Motility Assay

The mean surface area of agarose cell suspensions following antimetabolite treatment is depicted in figures 14 and 15. Statistical comparison using the Student *t* test failed to demonstrate a significant ($p < 0.05$) difference between serum containing, antimetabolite-free control wells and all other treatment wells.

Discussion

Dose Inhibition of cell Proliferation

Results from this study support the conclusion that a dose dependent inhibition of fibroblast growth occurs following exposure to either 5FU or MMC over all treatment intervals studied. Similar results have been previously reported using cultured rabbit,[13,16,17] and human[14,15] subconjunctival fibroblasts. Of these, only

one study[15] evaluated the effects over more than one time interval. MMC was not evaluated in this latter trial. From the present study it appears that canine fibroblasts demonstrate a similar response following short-term antimetabolite exposure. It has long been suspected, but never proven, that a similar response to low-dose long-term antimetabolite therapy might be achieved using a short-term high concentration antimetabolite protocol.[18] Results depicted in figure 5 confirm that the minimum concentration of antimetabolite required to induce a statistically significant reduction in fibroblast growth relative to time-matched control cells does decrease with increasing duration of exposure. It was also discovered that a similar inhibitory result was achieved with either antimetabolite if MMC concentrations were 1/10th that of 5FU. This ratio of potency was maintained over all treatment durations. Yamamoto et al. demonstrated the inhibitory effect of MMC to be 100 times that of 5FU when tested for 5 days on rabbit subconjunctival fibroblasts.[16]

The progression of cytologic changes observed in this study included cytoplasmic vacuolation, loss of cellular processes with resultant rounding of the cell, and finally disappearance of the cell from the monolayer. Similar observations have been reported using cultured rabbit[13, 16,17] and human[14,15] subconjunctival fibroblasts. Also described in one study using human subconjunctival fibroblasts,[14] was the observation that treatment with sublethal concentrations of antimetabolites (5FU 1 x 10^{-2} mg/l; MMC 1 x 10^{-3} mg/l) resulted in "arrested" total growth rates with preservation of cytologic integrity. This "arrest" persisted for the duration of all study periods. Such observations might suggest multiple routes of cytopathic toxicity which are somewhat dose dependent.

Reversal of Drug-Induced Growth Inhibition

Results from the reversibility of drug-induced growth inhibition study revealed that the inhibitory effects of MMC and 5FU, when applied at 1 x 10^{-2} mg/l, were maximized between 4 and 7 days of therapy and were no longer significantly different from 14 day continuous treatment values. Increasing the concentration of 5FU to 1 x 10^{-1} mg/l decreased this value to 2 days of therapy. Paralleling these results was a previous study involving cultured rabbit subconjunctival fibroblasts. There it was determined that the inhibitory effects of 5FU

and MMC were the same following 7 or 14 days of treatment, thereby suggesting that effects were maximized after 7 days.[16] When correlated to *in vivo* studies using 5FU in combination with assorted filtration procedures, it has also been observed that intensive therapy was not necessarily required in the second postoperative week in order to inhibit fibroblast proliferation.[2,19] More recent cell culture studies have demonstrated prolonged inhibition of human fibroblastic proliferation after a single 5FU exposure.[20] When compared with fibroblasts exposed to MMC (0.1 mg/ml) in which growth is arrested for 36 days, fibroblasts exposed to 5FU (25 mg/ml) began to proliferate after 12 days.[20] According to Tahery and Lee[21] natural alkaloids, including antibiotics such as bleomycin, doxorubicin and mitomycin may, even with short exposure times, have the ability to "suppress cellular proliferation completely" since their activity is relatively cell cycle non-specific when compared with 5FU. Furthermore, high concentrations of MMC may result in a cytocidal effect in addition to inhibition of fibroblast proliferation.[11] Numerous clinical studies support the efficacy of a single perioperative treatment of MMC in glaucoma patients undergoing filtration procedures.[6,9,10,22] Interestingly, when tissue levels of MMC were measured following a single intraoperative treatment, values fell well below the minimum antiproliferative level within a few days.[19] Half lives of MMC and 5FU have both been shown to range from 0.18 to 0.45 hours depending on the periocular tissue sampled.[19] It would follow that MMC's reportedly favorable antiproliferative activities last much longer in the clinical situation than the tissue concentrations would suggest.

Localization of F-Actin

F-actin is recognized as a major intracellular contractile protein involved in the cytoskeleton of cells. It has been suggested that this protein functions to mediate cell to substance adhesion through the attachment of polymerized F-actin to vinculin-containing cell membrane plaques.[23] During cell migration, bundles of F-actin form intracytoplasmic stress fibers (fig. 12) which extend into the newly formed cell processes providing the required tensile forces.[24] It has been postulated that the inhibitory effects of antimetabolites on tissue fibroblasts may result from an inhibition of F-actin monomer synthesis with resultant impaired polymerization

and attachment to the vinculin containing cellular membrane plaques.[2] Unlike a previous report of F-actin structure in rabbit subconjunctival fibroblasts stained with rhodamine phalloidin which showed no visible alterations following antimetabolite exposure,[16] results from this study reveal a clearly discontinuous linear pattern of fluorescein retention throughout the cytoplasm (fig. 13) The appearance of this feature prior to the loss of cytoplasmic processes implies that normal cell morphology is temporarily maintained after the polymerization of F-actin monomeric proteins has been affected.

Agarose Droplet Motility Assay

Fibroblast migration has been shown to be enhanced by several secreted chemoattractants such as fibronectin.[25] In this study we measured both serum-enhanced and drug-inhibited fibroblast migration using the agarose droplet motility assay. Results of our study failed to demonstrate a significant anti-motility effect from 5FU or MMC on treated fibroblasts over the 7 days of exposure. These results are in agreement with an earlier study which evaluated the effects of 5FU on rabbit subconjunctival fibroblasts.[16] From these two studies we conclude that these antimetabolites do not have a significant effect on fibroblast migration. Conversely, a wide variation in cell suspension diameter was observed within each treatment period as evidenced by the standard error of the mean bars (fig. 14 & 15). Increased sample sizes and serial replication could reduce these error values and reveal a significant trend.

Summary

There are limitations when applying cell culture findings to *in vivo* therapy in eyes undergoing glaucoma filtration surgery. The cells are monolayers growing on plastic, as opposed to cells in a tissue, and the majority of cells are actively proliferating under the influences of serum, a factor which may make them more susceptible to the effects of antiproliferative agents.[15] Antimetabolite perfusion is another important variable. In a recent report of rabbits undergoing full-thickness glaucoma filtering surgery combined with an antimetabolite protocol developed from an earlier *in vitro* model, reproliferation of clusters of relatively unaffected fibroblasts was observed following MMC therapy. This was likely due to inadequate drug

concentrations in some cells as a result of impaired penetration of subconjunctival tissues during the 5 minute exposure period.[13] Nevertheless, these studies do provide the framework for continued research in the clinical setting. In the present study, canine fibroblasts have been shown to respond to 5FU and MMC in a manner similar to human and rabbit fibroblasts. Future canine clinical trials combining antimetabolite therapy with appropriate surgical procedures could yield the same favorable results as are being experienced in human glaucoma patients. The authors recommend continued research into this area.

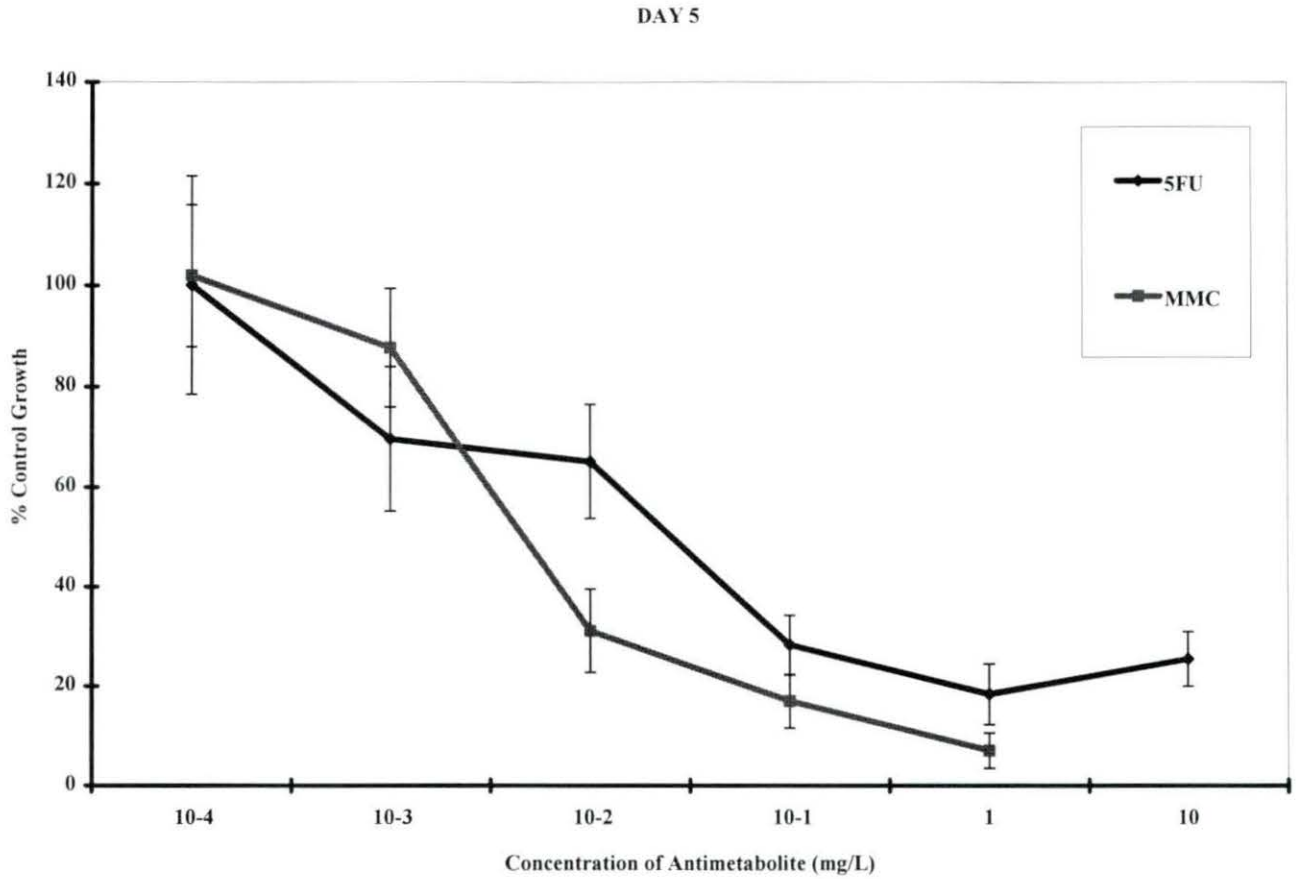

Fig. 1 Dose Inhibition Study of Cell Proliferation. Number of cells observed from antimetabolite-treated wells, expressed as a percentage of drug-free control well values. Vertical bars with brackets indicate standard error of the mean.

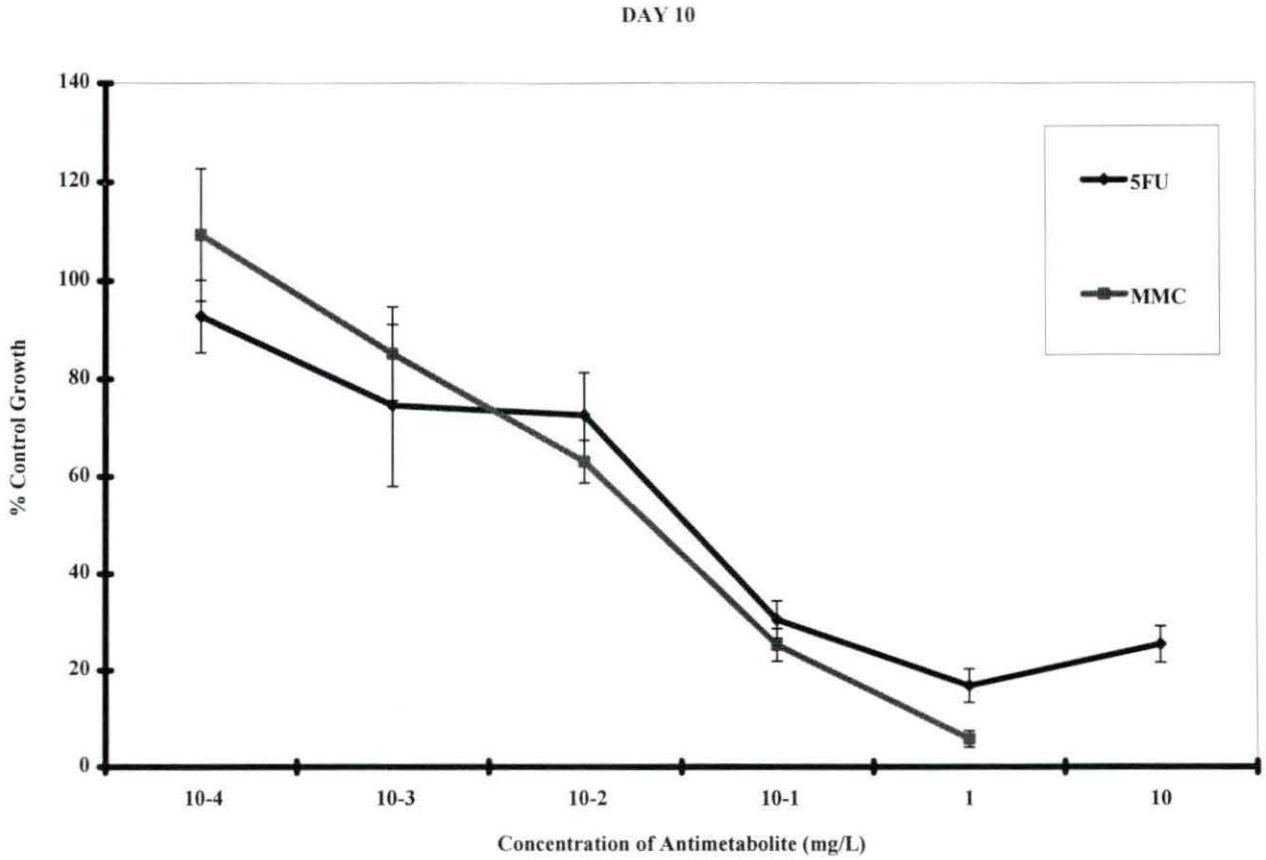

Fig. 2 Dose Inhibition Study of Cell Proliferation. Number of cells observed from antimetabolite-treated wells, expressed as a percentage of drug-free control well values. Vertical bars with brackets indicate standard error of the mean.

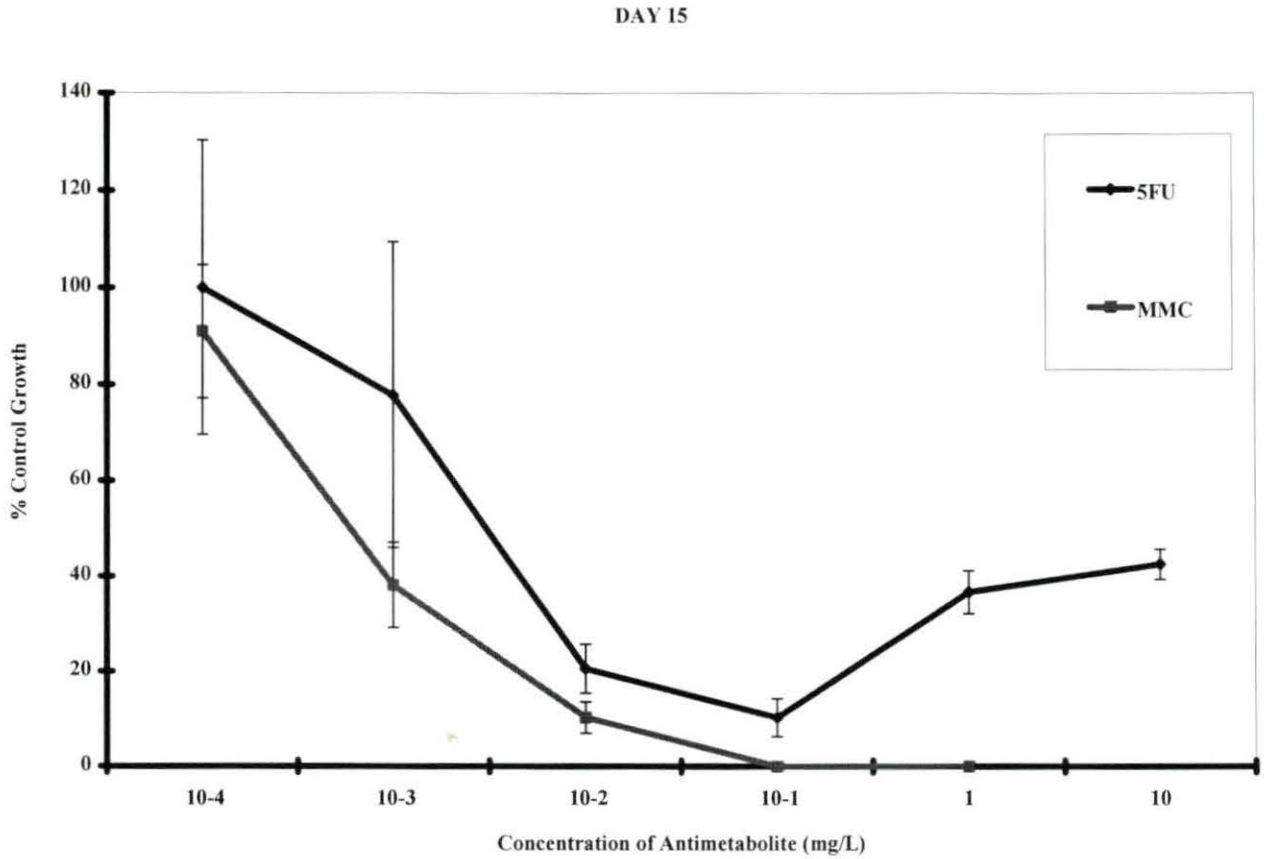

Fig. 3 Dose Inhibition Study of Cell Proliferation. Number of cells observed from antimetabolite-treated wells, expressed as a percentage of drug-free control well values. Vertical bars with brackets indicate standard error of the mean.

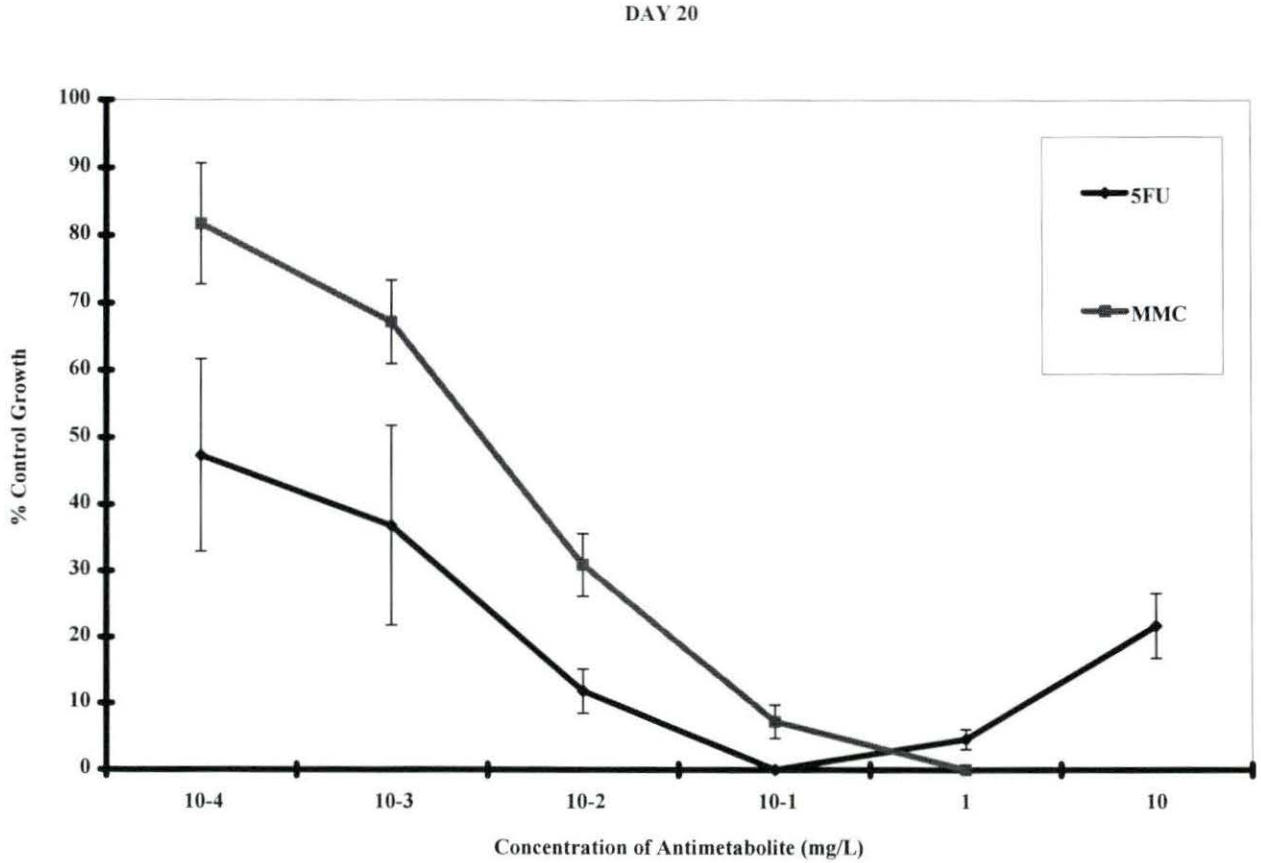

Fig. 4 Dose Inhibition Study of Cell Proliferation. Number of cells observed from antimetabolite-treated wells, expressed as a percentage of drug-free control well values. Vertical bars with brackets indicate standard error of the mean.

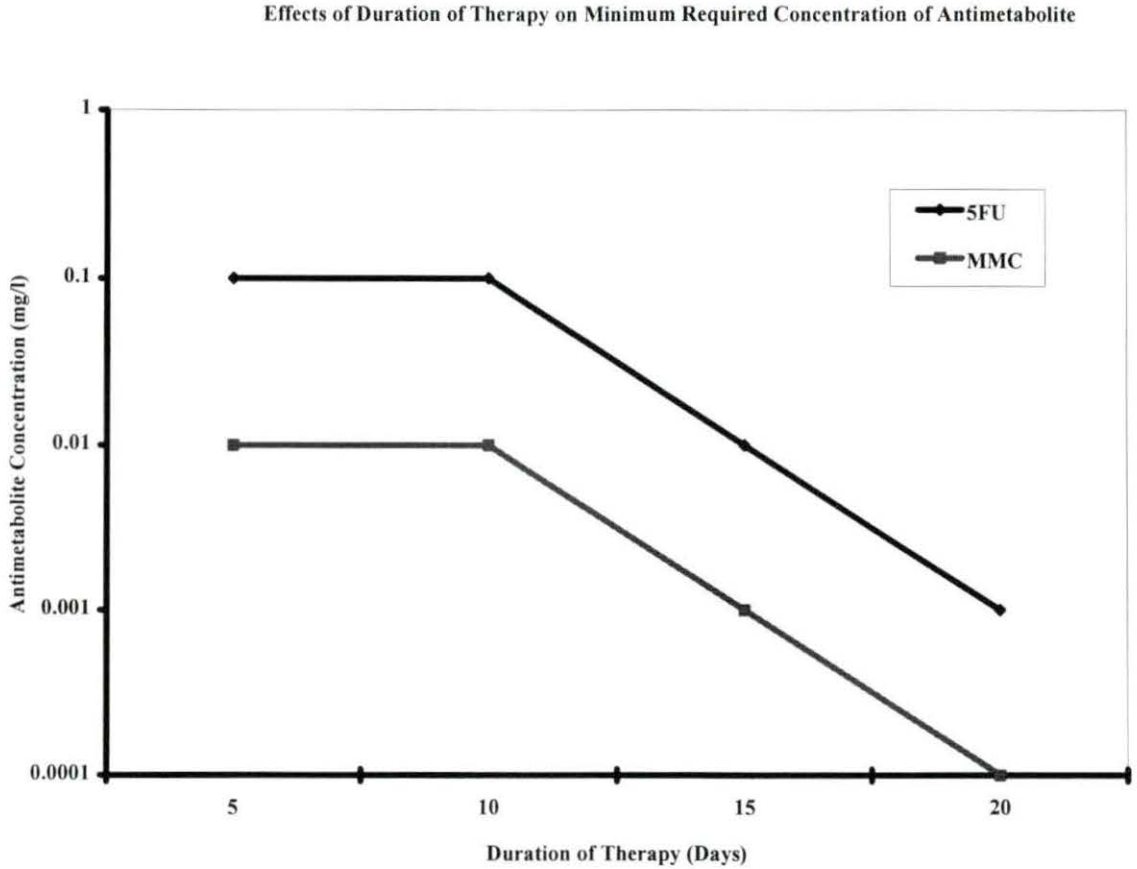

Fig. 5 Minimum Concentration of 5FU and MMC required to yield significant ($p<0.05$; Student *t* test) reduction in fibroblast growth relative to control wells following 5, 10, 15 or 20 days of therapy.

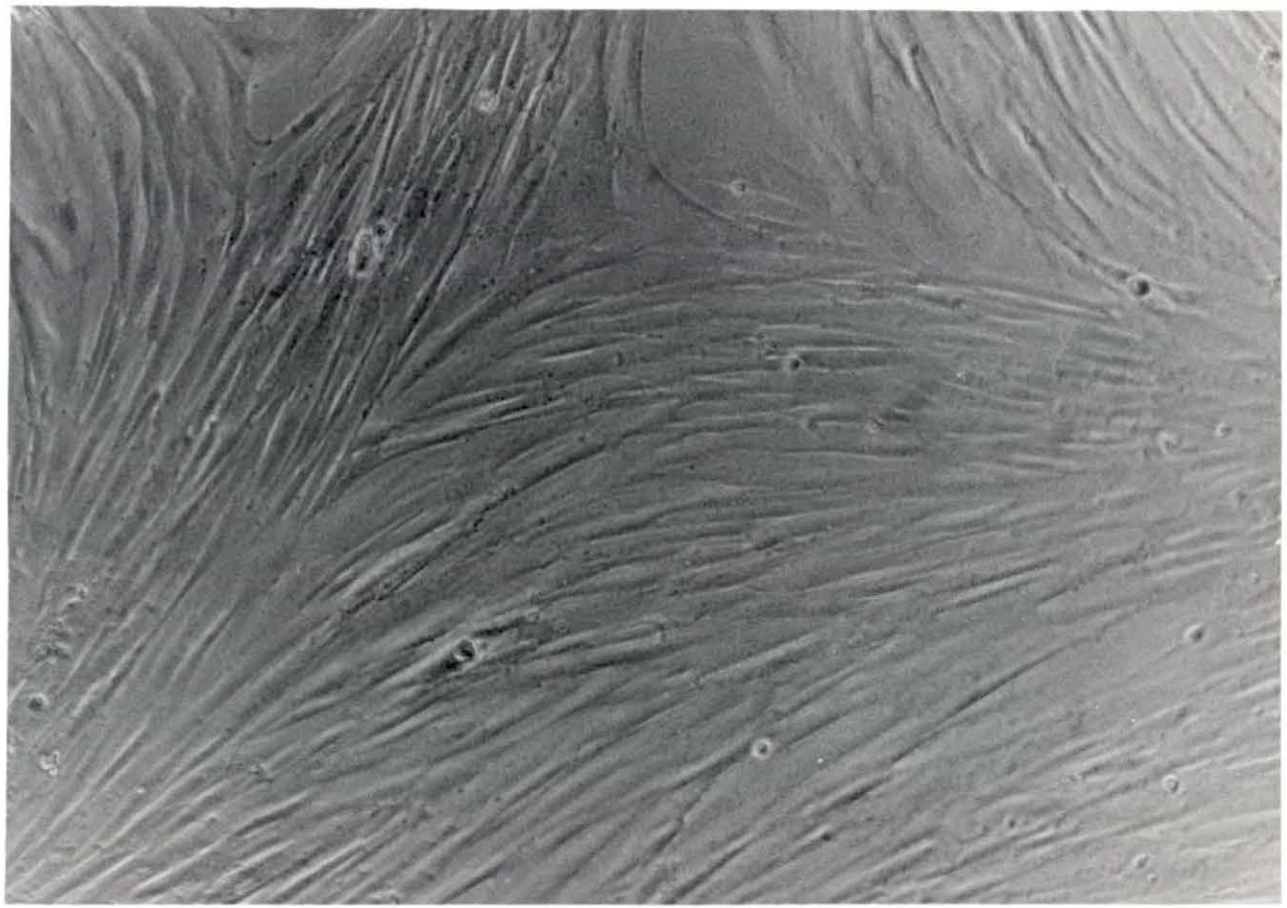

Fig. 6 Fibroblasts following 5 days of growth (control well). Note the uniform monolayer of cells. (x125)

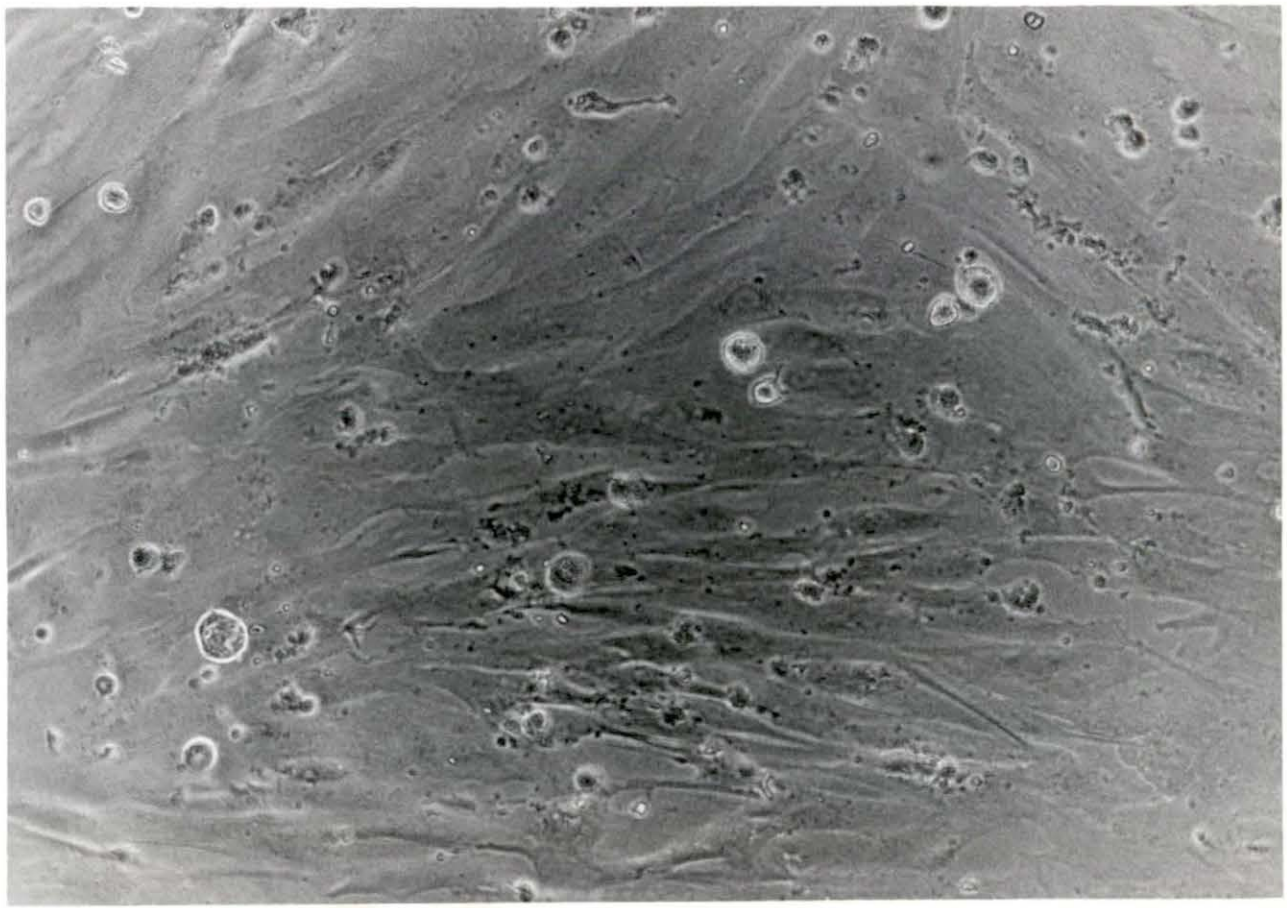

Fig. 7 Fibroblasts following 3 days of treatment (5FU 1 x 10^{-2} mg/l). Note the increased cytoplasmic granularity and early rounding of cells. (x312)

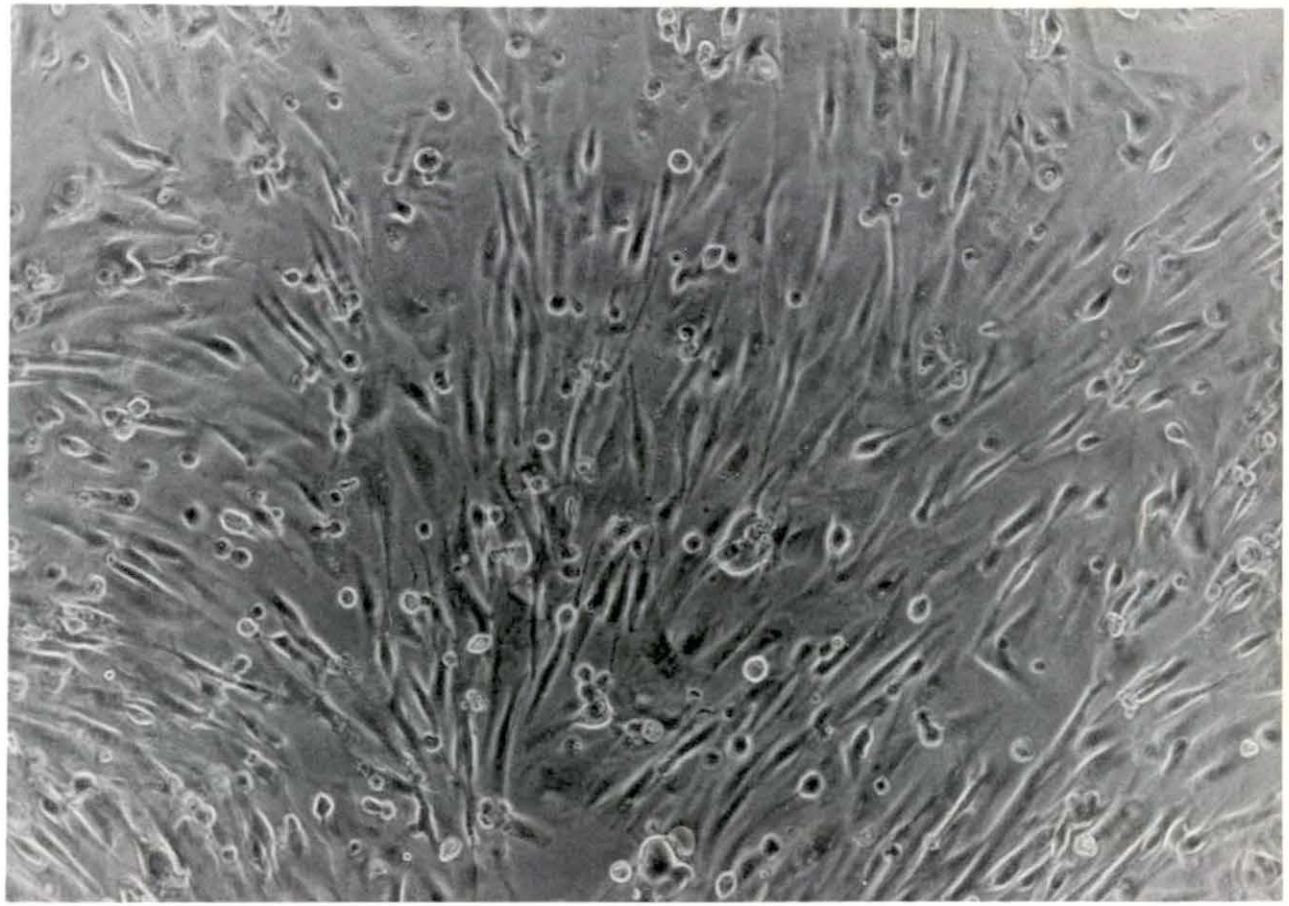

Fig. 8 Fibroblasts following 5 days of treatment (5FU 1 x 10^{-2} mg/l). Moderate rounding of affected cells with resultant gaps in the monolayer is observed. (x125)

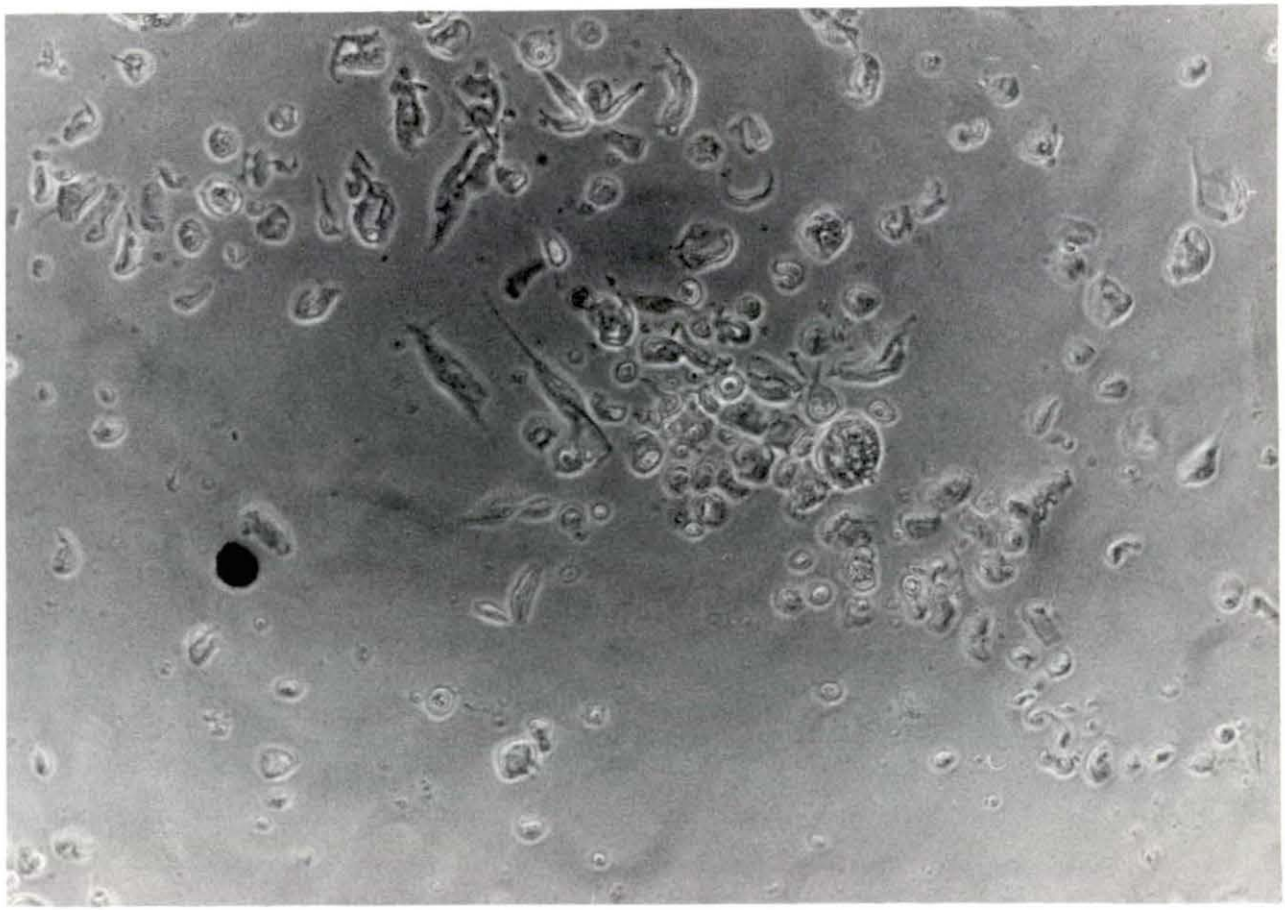

Fig. 9 Fibroblasts following 13 days of treatment (MMC 1 x 10^{-2} mg/l). Note the virtual absence of spindle shaped cells with residual fibroblasts being uniformly rounded. (x175)

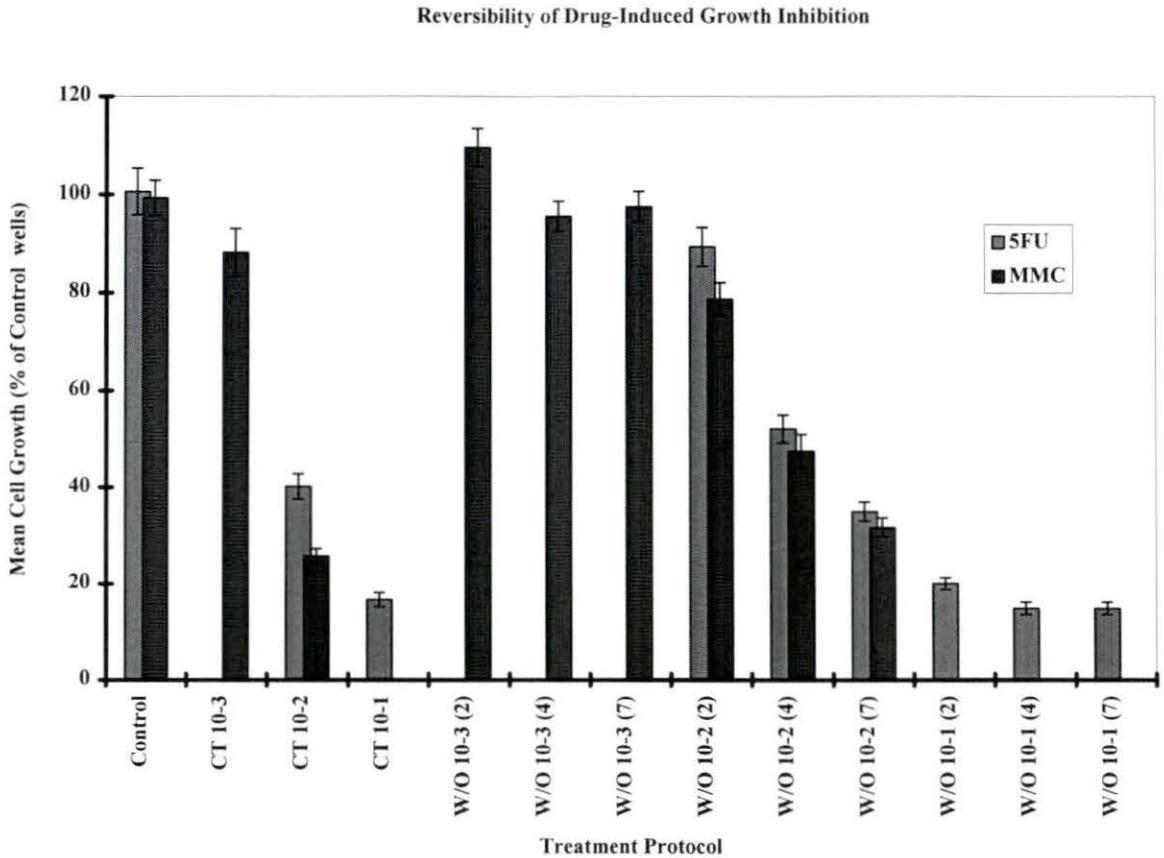

Fig. 10 Graph depicts the relative growth of antimetabolite treated fibroblasts as a percentage of the mean growth of cells from control wells. Legend: CT (continuous treatment), W/O (washout), 10-n (1 x 10^{-n} mg/l), 2, 4, or 7 = duration of antimetabolite exposure prior to washout

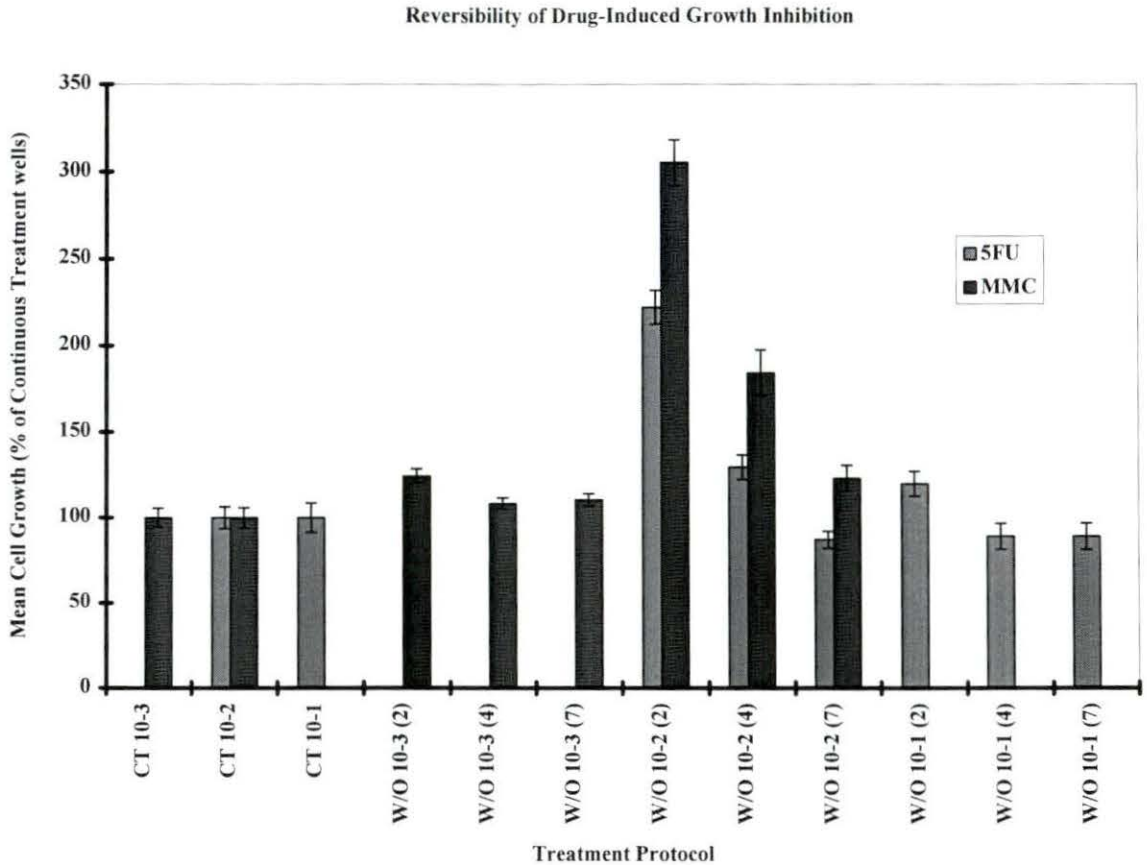

Fig. 11 Graph depicts the relative growth of antimetabolite treated fibroblasts as a percentage of the mean growth of cells from antimetabolite matched continuous treatment wells. Legend: CT (continuous treatment), W/O (washout), 10-n (1 x 10^{-n} mg/l), 2, 4, 7 = duration of antimetabolite exposure prior to washout

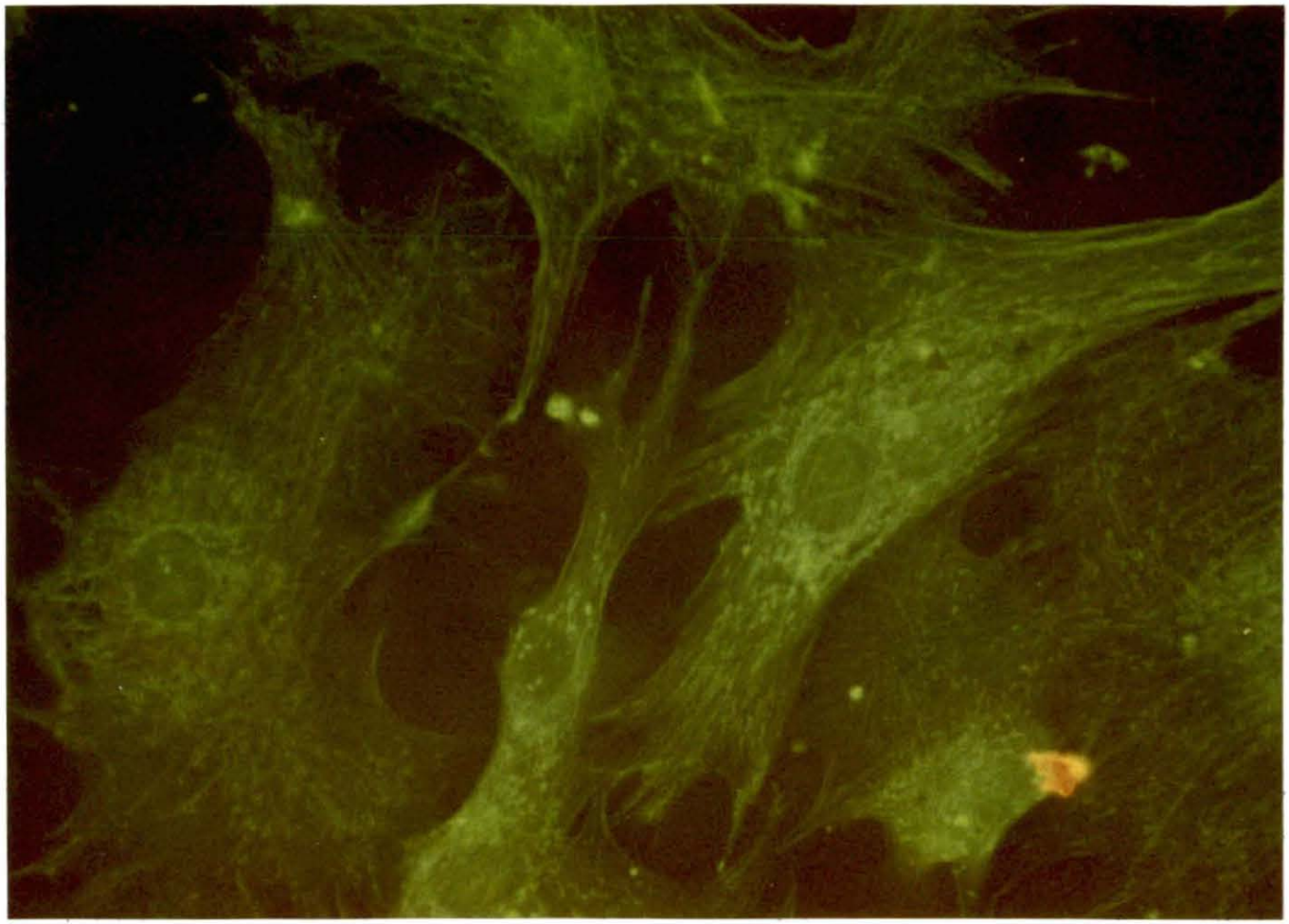

Fig. 12 Fluorescein labeled F-actin filaments are observed as continuous bands extending throughout the entire cytoplasm. (control well) (x510)

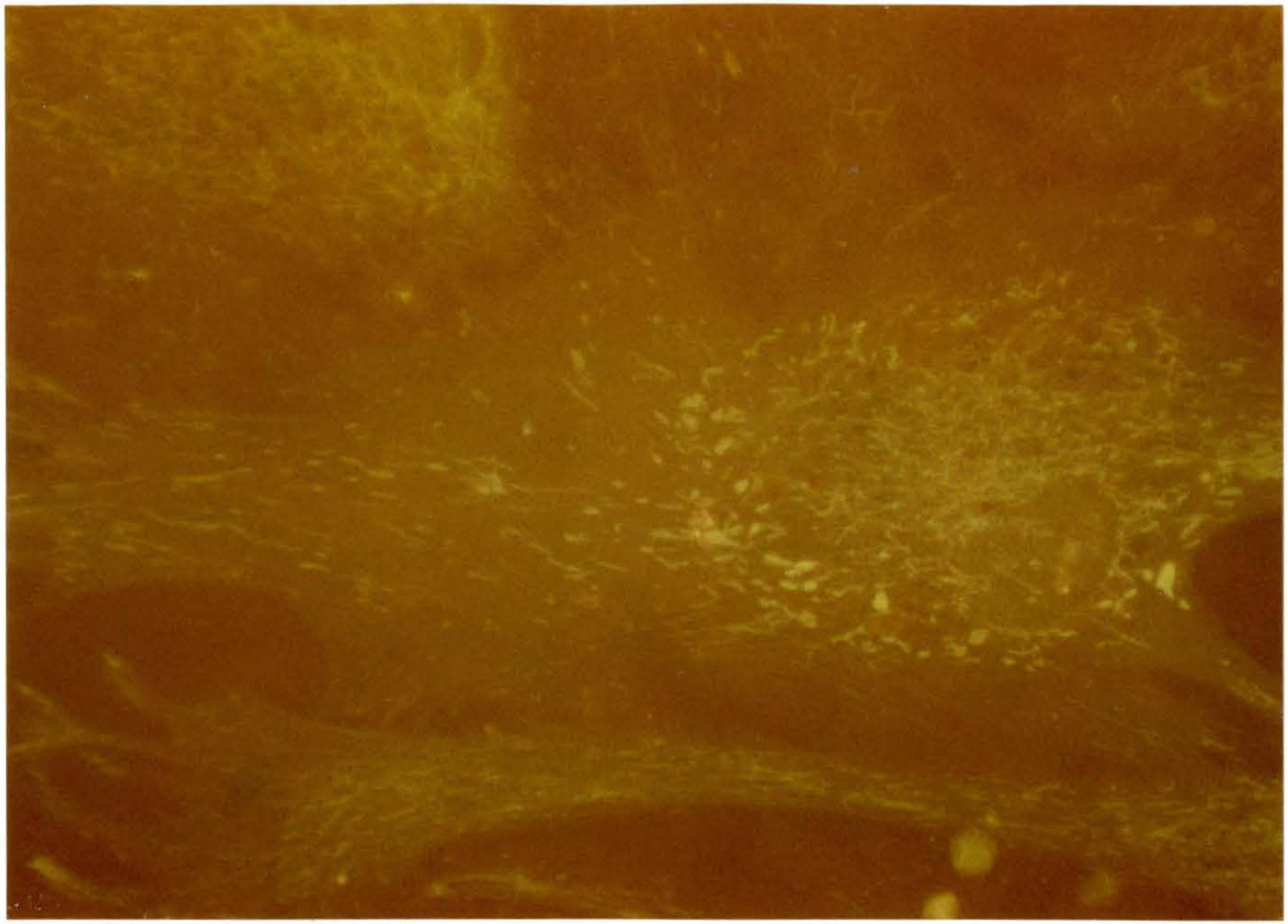

Fig. 13 Fluorescein labeled F-actin following 5 days of treatment (MMC 1 x 10^{-3} mg/l). Filaments are observed in a discontinuous linear pattern extending throughout the cytoplasm. (x780)

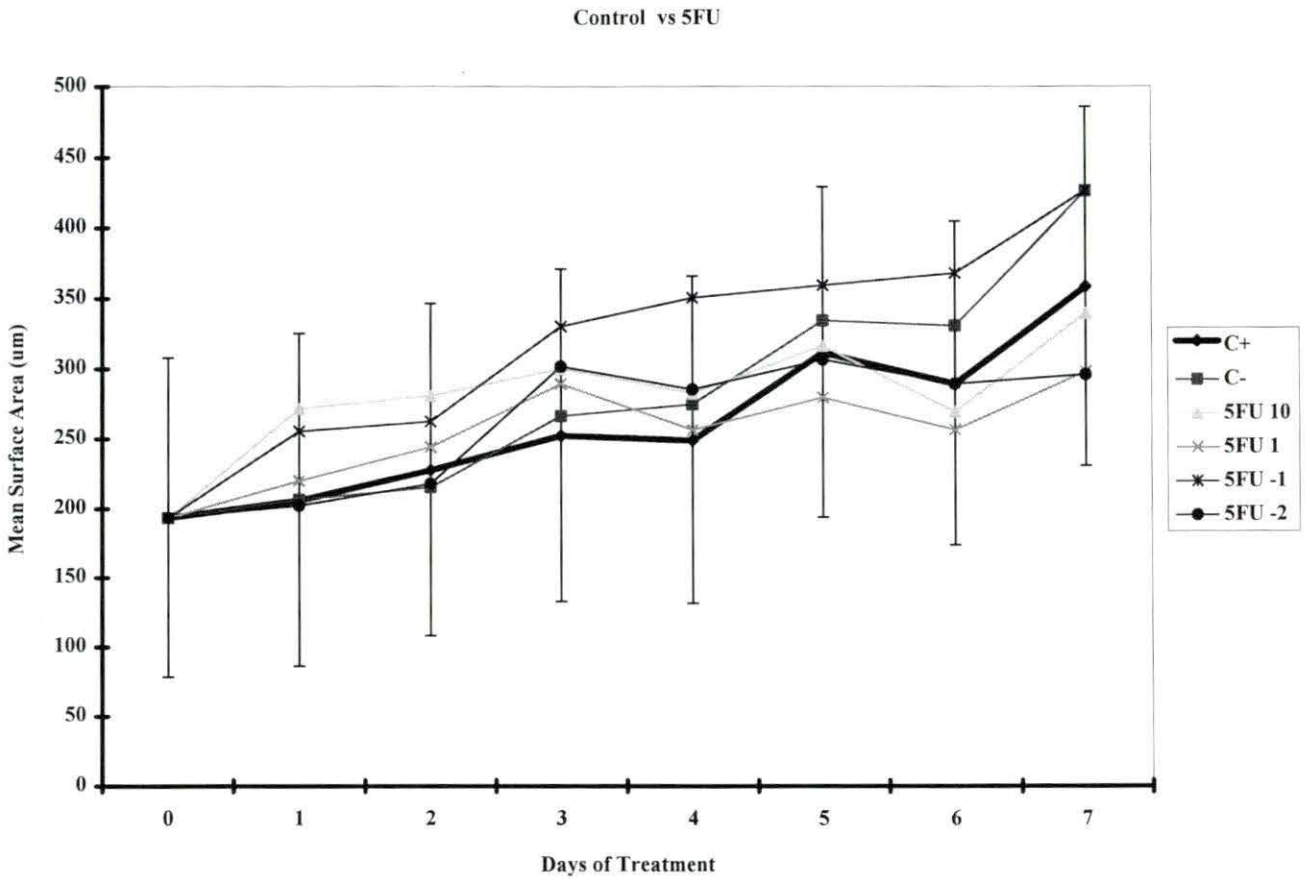

Fig. 14 Agarose Droplet Motility Assay Comparison of Surface Area of Growth (Control vs. 5FU). Legend: C + = (bold line graph; control wells with serum), C – = (control wells without serum), 5FU 10, 1 -1, -2 (5FU at 1 x 10 (number) mg/l). Vertical bars with brackets indicate standard error of the mean for the serum containing control series.

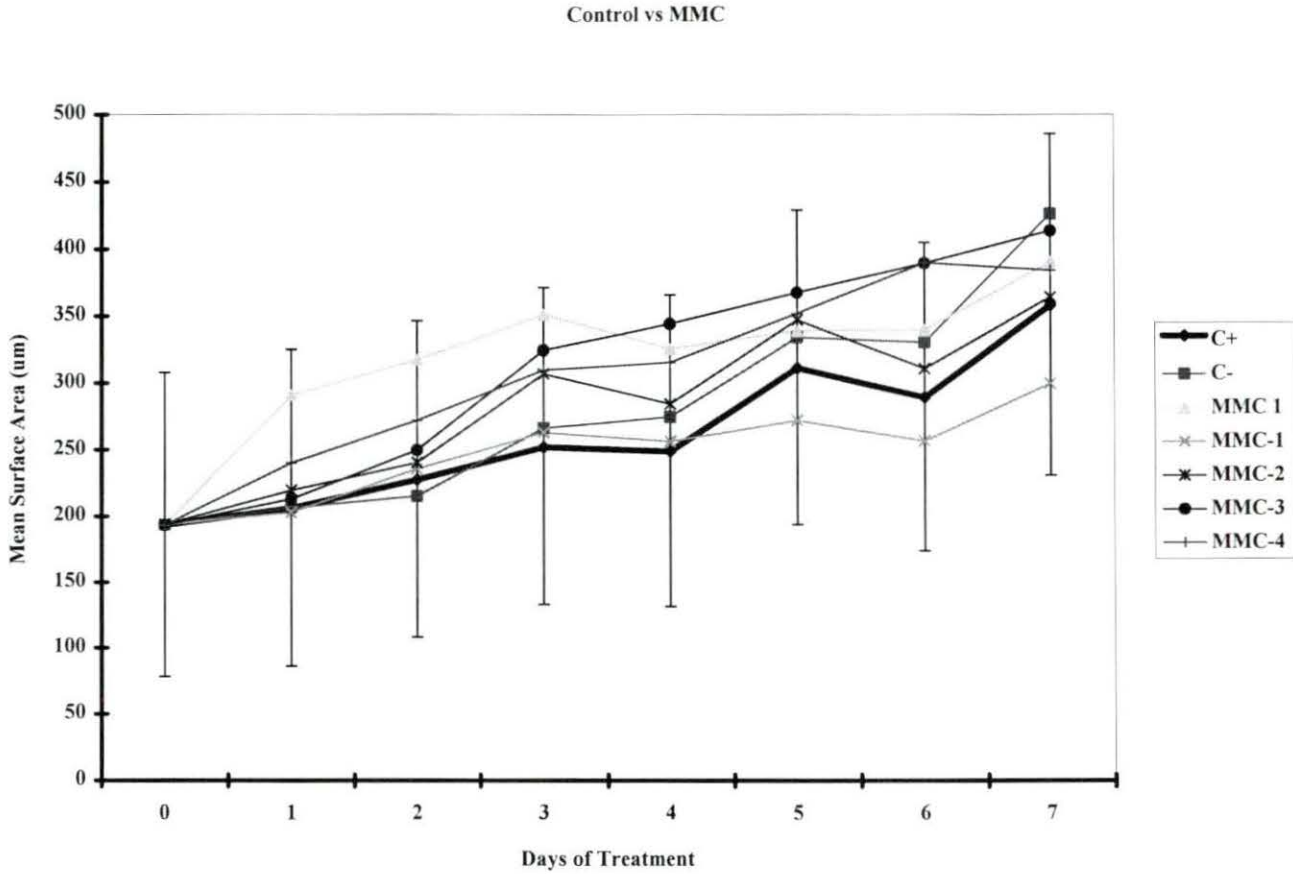

Fig. 15 Agarose Droplet Motility Assay Comparison of Surface Area of Growth (Control vs. MMC). Legend: C + = (bold line graph;control wells with serum), C – (control wells without serum), MMC 1 -1, -2 (MMC at 1 x 10 (number) mg/l). Vertical bars with brackets indicate standard error of the mean for the serum containing control series.

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3. *IN VIVO* CLINICAL TRIAL OF PERIOPERATIVE MITOMYCIN-C IN COMBINATION WITH BAERVELDT DRAINAGE DEVICE IMPLANTATION IN NORMOTENSIVE CANINE GLOBES

A paper to be submitted to Veterinary & Comparative Ophthalmology

D. Tinsley, Y. Niyo, D. Betts

Abstract

The authors evaluated the effects of mitomycin C (MMC) exposure on normotensive canine globes undergoing glaucoma seton surgery. The twelve dogs examined in this study were randomly assigned into one of three groups of four individuals with each group being monitored for 1, 2, or 3 months. One eye of each dog was randomly assigned to the MMC treatment group with the fellow eye serving as the control. Numerous clinical differences were observed. While 12/12 MMC treated eyes maintained blebs of uniform distention, 9/12 control eyes became tightly adherent to the underlying sclera anterior to the scleral footplate. Bleb pallor was observed in 10/12 MMC treated eyes while all control blebs maintained a uniform pattern of conjunctival vascularization. Also observed was conjunctival microcyst formation (1/12 control eyes; 3/12 MMC treated eyes) and corneal overriding (0/12 control eyes; 2/12 MMC treated eyes). Intraocular complications included transient collapse of the anterior chamber (2/12 control eyes; 7/12 MMC treated eyes) with resultant tube induced dyscoria (1/12 control eyes; 3/12 MMC treated eyes) and focal corneal edema (2/12 MMC treated eyes). Single occurrences of cataract formation and anterior vitritis with peripapillary retinal detachment was identified in MMC treated eyes. Serial tonometry of dogs monitored for 30 days (n=12) or 61 days (n=8) showed significantly ($p<0.01$) reduced intraocular pressures (IOP) in MMC treated eyes. No significant ($p< 0.05$) difference in postoperative tonography values was recorded between control and MMC treated eyes. Filtration bleb capsules from MMC treated eyes were thinner ($p< 0.05$) with a looser arrangement of collagen fibers than those recorded from control eyes. Comparison of fibroblast outgrowth between MMC treated and control explants revealed significant ($p< 0.0001$) reduction following previous antimetabolite therapy. This finding parallels a previous *in vitro* study by the current authors. Interpretation of the present study supports extended viability of filtration bleb with prolonged IOP regulation following MMC therapy, but with an

increase incidence of transient intraocular complications. Recent clinical trials by the authors using a reduced dosage of MMC in canine glaucoma patients has demonstrated an absence of clinical complications while maintaining filtration bleb viability and IOP regulation.

Introduction

Surgical management of human open-angle glaucoma patients is generally reserved for those individuals who have been non-responsive to traditional medical therapy. Most physicians have adopted a regimen of surgical procedures. Argon laser trabeculoplasty represents the initial procedure of choice. Poorly responsive patients typically undergo a surgical trabeculectomy. In the event that this is also unsuccessful, continued management efforts include either seton implantation or a ciliodestructive procedure.[1] While advent of the Nd:YAG laser has permitted development of a new avenue of cyclophotocoagulation, it has not been without complications. Recent studies have shown that many patients require this procedure to be repeated at least once in order to maintain ocular normotension. Even when it is designated "effective", human patients continue to require more anti-glaucoma medications than similar individuals undergoing glaucoma seton surgery.[2]

Veterinary literature documents the historic use of numerous filtration procedures. Included are iridencleisis, cyclodialysis, peripheral iridectomy, combined iridencleisis-cyclodialysis, corneoscleral trephination-peripheral iridectomy, cyclodialysis-iridocyclectomy, and posterior sclerectomy-cyclodialysis-transscleral iridencleisis.[3,4] One of the new and evolving areas of glaucoma research is the use of anterior chamber drainage devices. A wide variety of implants are currently being used for glaucoma management in man[1,5-8] and the dog. [9-13] All incorporate a hollow silicon tube which functions as a sclerostomy opening, shunting aqueous humor to the adjoining polypropylene or silicon foot plate (scleral explant). Emergent fluid collects in the surrounding filtering bleb and is taken up by the regional vascular channels.[11] Following

implantation, the developing fibrosis at the site of the filtration bleb acts as the main regulator of intraocular pressure (IOP).

The most widely reported complication associated with both seton and glaucoma filtration surgeries is scarring of the resultant filtration bleb following proliferation of subconjunctival fibroblasts and biosynthesis of collagen and other related extracellular materials.[7,14,15] This complication has been especially pronounced in young patients.[16] In an effort to retard this fibroplasia, numerous antimetabolites have been incorportated into the surgical or postoperative medical protocol. Mitomycin C (MMC) is an anti-tumor antibiotic isolated from *Streptomyces caespitosus*. Its anti-tumor effect is attributed to the inhibition of DNA replication leading to cell death.[17] Mitomycin C alkylates and crosslinks DNA, inhibits DNA, RNA and protein synthesis, and in addition, may generate superoxide and hydroxyl radicals.[18] The combination of these features are believed to be responsible for the clinically observed long-term inhibitory effects on fibroblast proliferation.[8,17,19-21] Additional *in vitro* studies evaluating the effects of MMC on cultured human, primate and rabbit subconjunctival fibroblasts have demonstrated a significant inhibitory effect on cell growth and replication.[14,22-25] In an earlier study[26] the present authors investigated the inhibitory effects of MMC and 5FU on cultured canine subconjunctival fibroblasts. Results paralleled those reported from other species in that inhibition was found to be both time and dose dependent.

There are limitations when applying cell culture findings to *in vivo* therapy in eyes undergoing glaucoma filtration or seton surgery. The cells are monolayers growing on plastic, as opposed to cells within a tissue, and the majority of cells are actively proliferating under the influences of serum, a factor which may make them more susceptible to the effects of antiproliferative agents.[25] Antimetabolite perfusion is another important variable. In a recent report of rabbits undergoing full-thickness glaucoma filtering surgery combined with an antimetabolite protocol developed from an earlier *in vitro* model, reproliferation of clusters of relatively unaffected fibroblasts was observed following MMC therapy. This was due to inadequate antimetabolite

concentrations in some cells from impaired penetration through subconjunctival tissues during the 5 minute exposure period.[23]

The purpose of this study was to examine the effects of single dose MMC therapy on normotensive globes undergoing glaucoma seton surgery. Effects were monitored using routine clinical evaluation, tonography and serial tonometry. At the termination of the clinical trial, all globes and filtration blebs were removed for histologic examination. Tissue explants from each filtration bleb were harvested for subsequent fibroblast culture studies. Growth rates of MMC treated fibroblasts were compared to similar cells from control eyes. The authors were unable to identify similar clinical trials in the human or veterinary literature.

Materials and Methods

Twelve young to middle aged, medium sized (9.4 to 17.6 kg) mixed-breed dogs (8 males, 4 females) were obtained for this study. No systemic abnormalities were observed on general physical examination, routine fecal flotation or preliminary complete blood cell counts. All individuals were determined to be free of ocular abnormalities using slit lamp biomicroscopy (Kowa SL-2, Kowa Co. Ltd., Tokyo, Japan), indirect ophthalmoscopy (Keeler Inst. Co. Inc., Broomall, PA), gonioscopy (Franklin goniolens, Parsons Optical, San Francisco, CA) and applanation pneumotonography (Alcon Applanation Pneumotomograph,™ Alcon Surgical Inc., Fortworth, TX). Dogs were randomly assigned into one of three groups of four individuals, with each group being evaluated for 1, 2 or 3 months duration. All dogs were acclimated for 10 days prior to the initiation of the study.

Dogs were medicated perioperatively with a topical gentamicin sulfate (Gentocin,® Schering-Plough Animal Health, Kenilworth, NJ)/ dexamethasone (Azium,® Schering-Plough Animal Health, Kenilworth, NJ) solution, oral amoxicillin (Amoxi-tabs,® SmithKline Beecham Animal Health, West Chester, PA) and oral prednisone (Prednisone, Roxanne Labs. Inc., Columbus, OH) according to the protocol listed in table 1. All

dogs were sedated using 0.5 mg/kg IV acepromazine maleate (PromAce,® Fort Dodge Labs. Inc., Fort Dodge, IA) followed five minutes later with 10 mg/kg IM ketamine hydrochloride (Ketaset,® Fort Dodge Labs., Fort Dodge, IA) prior to performing tonography.[27] Coefficient of aqueous outflow was determined for each eye using 4 minute tonography values obtained using an applanation pneumotomograph (Alcon Surgical Inc., Fortworth, TX). General anesthesia was subsequently induced using 5 mg/kg IV thiopental sodium (Fluothane,® Abbott Labs., Chicago, IL), and maintained with inhalant halothane (Halothane,® Abbott Labs. Inc., Abbott Park, IL).

Following routine surgical preparation, Steven's tenotomy scissors were used to create a full thickness fornix based conjunctival flap with the initial incision 5 mm caudal to and parallel with the dorsolateral limbus, portending an arc of 120-140 degrees. Sharp and blunt dissection extended the cavity caudally in the subtenon's space until good visualization of the superior and lateral rectus muscles was achieved. The respective muscle bodies were freed from their underlying fascial attachment in order to create a site for future insertion of the scleral explant. Prior to its positioning, each subtenon's pocket was randomly treated with either 0.5 mg/ml mitomycin C (MMC) (Mutamycin,® Bristol Labs., Princeton, NJ) (n=12) or a physiologic saline placebo (n=12). Two ophthalmic sponges (Microsponge,® Alcon Labs. Inc., Fort Worth TX), each soaked with 0.3 ml of either physiologic saline or MMC, were placed deep within the subtenon's space of each eye and allowed to remain for 5 minutes. The sponges were then removed and the entire surgical field was copiously irrigated with 125 ml of physiologic saline.

The footplate of the drainage device (Baerveldt, ® model AG101-350mm^2, Iovision Inc., Irvine, CA) was positioned sclerad to the superior and lateral rectus muscles and affixed to the sclera with two ligatures of 9-0 polypropylene (Prolene,® Ethicon Inc., Sommerville, NJ). Following sharp dissection anteriorly to the limbus, a 21 gauge hypodermic needle was used to enter the anterior chamber from a site 1mm caudal to the surgical limbus. The needle tract, which would later be used for insertion of the drainage tube, was directed midway between the inner corneal surface and the anterior iris face. Prior to its insertion, the drainage tube was

trimmed so as to project 5 mm from the iridocorneal angle. Once inserted, the tube was secured to the sclera 4 mm caudal to the limbus using a single horizontal mattress suture of 9-0 polypropylene. Tenon's capsule and the overlying conjunctiva were meticulously closed in two layers using 7-0 polyglactin 910 (Vicryl,® Ethicon Inc., Sommerville, NJ). The anterior chamber was reformed with a lactated ringers solution using a 30 gauge needle inserted through the cornea 1mm from the limbus. A subconjunctival injection of 0.2 ml of betamethasone (Betasone,® Schering -Plough Animal Health, Kenilworth, NJ) was given in the ventronasal quadrant, adjacent to the limbus, of all operated globes.

Postoperative examinations of all globes and filtration blebs (including IOP's) were performed 3 times weekly for the duration of the study. Adnexal, anterior segment, and fundic photographs were obtained using a Kowa RC-2 fundus camera (Kowa Co. Ltd., Tokyo, Japan). At the termination of the study (1, 2, or 3 months), dogs were again sedated, tonography was performed, and the individuals euthanatized (Beuthanasia-D,® Schering -Plough Animal Health, Kenilworth, NJ). Immediately afterwards, the entire dorsal bulbar conjunctiva was surgically removed and a 1 cm x 1 cm full thickness calotte of subconjunctival tissue including tenon's capsule was removed and transferred into a sterile petri-plate containing minimal essential media (MEM) (Gibco Biologics, Grand Island, NY) for transfer to the cytology lab. Each globe and associated filtration bleb was then enucleated and placed in either formalin or Bouins solution. Tissues were processed using Hematoxylin and Eosin and Trichrome stains for subsequent histologic examination.

Harvested subconjunctival tissues were divided into multiple 2 mm x 2 mm blocks and placed in 40 ml (75 sq. cm) tissue culture flasks (Costar Plastics, Rochester, NY) with MEM fortified with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO), gentamicin sulfate (25 mg/500 ml MEM) (Gentocin,® Schering-Plough Animal Health, Kenilworth, NJ), and Amphotericin B (1 mg/500 ml MEM) (Fungizone,® ER Squibb & Sons Inc., Princeton, NJ). Cells were incubated at 37°C in 5% CO_2 (Heinicke Co., Portland, OR) and allowed to reach confluence before being passaged. Media was replaced every 48 to 72 hours in all cell cultures. At the time of passage, media was decanted, cells were treated with a 0.05% trypsinizing solution (Gibco Biologics,

Grand Island, NY) and incubated for 5 minutes. The flask was gently agitated and the resultant suspension of cells was pipetted ("Pipet-aid", Drummond Scientific Co., Broomall, PA) to recipient flasks or tissue culture plates. A 0.25ml aliquot was stained using 0.4% trypan blue (Sigma Chemical Co., St. Louis, MO) and cell concentrations were manually determined using an Improved Neubaur Hemocytometer (Fisher Scientific, Pittsburgh, PA) and inverted microscopy (Leitz Diavert) (Henry Louis Inc., Iowa City, IA).

Results

Clinical Observations

Anterior chamber fibrin was noted in the immediate postoperative period in 3/12 control eyes and 4/12 MMC treated eyes. Although all eyes showed extension of the organizing fibrin into the drainage tube, maintenance of tube patency was implied by continued intraocular normotension and the presence of a visible filtration bleb (fig. 1). Throughout the first two weeks of each study group, no readily identifiable difference was observed between the filtration blebs of control and MMC treated eyes. All blebs were smoothly distended caudal to the site of the original conjunctival incision, while that portion of the conjunctiva anterior to the incision was in direct apposition to the underlying sclera (fig. 1). In two individuals the overlying tissues were sufficiently thin as to allow clear visualization of the polypropylene suture used to secure the drainage tube (fig. 1). A clinical dichotomy developed beyond postoperative day 14. With a mean time of initial observation of 24.8 ± 6.5 days, an increased distention of the filtration bleb was observed in 3/12 control eyes and 12/12 MMC treated eyes. Figure 2 illustrates the typical appearance of a MMC treated eye. The anterior conjunctiva and subconjunctival tissues appear more uniformly distended from the underlying sclera with the resultant filtration bleb extending anteriorly to the level of the limbus. While the control eye retained a visible filtration bleb caudal to the conjunctival incision, it's size and extent was significantly less than that of the MMC treated eye. Pallor of the filtration bleb was observed in 10/12 MMC treated eyes with a mean detection time of 20.0 ± 2.3 days (fig. 2). This finding was not observed in any of the control eyes. Slit lamp biomicroscopy revealed

microcyst formation in 1/12 control blebs and 3/12 MMC treated eyes (fig. 2). While not observed in any of the control eyes, 2/12 MMC treated eyes showed regions of the dorsal bulbar conjunctiva which protruded over the adjacent cornea (fig. 3).

The following intraocular abnormalities were recorded. Collapse or marked narrowing of the anterior chamber was identified in 2/12 control eyes and 7/12 MMC treated eyes (fig. 4). In the latter group, the mean time of initial observation was day 12.8 ± 1.4. Anterior chamber collapse was invariably associated with a sudden marked distention of the associated filtration bleb (fig. 4) along with mild to moderate blepharospasm and lacrimation. Conjunctival wound leakage was not observed clinically, or supported diagnostically through Seidel testing, in any of the operated eyes. All anterior chambers spontaneously reformed following a mean of 10.8 ± 2.2 days. Observed complications following reformation included persistent tube to iris contact with resultant dyscoria (1/2 control eyes; 3/7 MMC treatment eyes), and focal non-progressive endothelial based corneal edema (2/3 MMC treated individuals) secondary to tube touch. One additional abnormality not associated with anterior chamber collapse was a linear subcapsular cataract directly underlying the drainage tube (1/12 MMC treated eye). First observed on day 31, this cataract showed only minimal progression by postoperative day 95 (fig. 5). Posterior segment abnormalities were limited to anterior vitritis (MMC treated eye; Day 15) and marked peripapillary vascular engorgement (control eye; Day 82), with both occurring in the same dog. Subsequent histologic examination revealed a mild suppurative anterior vitritis and peripapillary retinal detachment of the MMC treated eye with no identifiable intraocular microorganisms. Evaluation of the control eye confirmed vascular congestion of the peripapillary area. Ophthalmoscopic examination of the temporal fundus of all operated globes demonstrated a hyporeflective quadrant of the tapetal fundus immediately underlying the scleral footplate (fig. 6). Despite a clinical similarity to a peripheral bullous retinal detachment, these findings were ophthalmoscopically non-progressive and later histologic examination showed normal apposition of the neurosensory retina.

Tonometry/ Tonography

When comparing the mean intraocular pressures (IOP's) of control and MMC treated eyes for the four dogs within any given treatment group (1, 2, or 3 months postoperative period), no significant difference was observed ($p < 0.05$; Student *t* test). When sample sizes were enlarged so as to include all animals being treated for 30 days (n=12), or 61 days (n=8), a significant ($p < 0.01$) reduction in IOP was observed in MMC treated eyes (figs. 7 & 8).

Tonographically derived coefficient of outflow ("C") values for control and MMC treated eyes following 1, 2 or 3 months of monitoring are depicted in figure 9. Although 9/12 individuals showed a trend towards having higher "C" values from control eyes than observed with MMC treated eyes, this trend was not significant ($p<0.05$) when comparing all dogs (n=12), or dogs within any particular treatment group (n=4) using the Student *t* test.

Gross Pathologic Evaluation

All tubes (n=24) were patent at the termination of the study. This finding was supported by the rapid collapse of all anterior chambers following surgical entry into the overlying filtration bleb as well as unimpeded saline irrigation of the tube lumen following removal from the globe. A subjective difference in the apposition of the fibrous capsule to the enclosed scleral explant was observed as tissues were being harvested for *in vitro* fibroblast culture. Eleven of twelve MMC treated eyes demonstrated loose capsular attachment to the implant while 9/12 capsules from control eyes were tightly adherent. This difference was observed within all three treatment groups (1, 2 or 3 months).

Histopathology

An average capsule thickness was determined from sixteen separate measurements made from two cross sectional samples of each filtration bleb. Mean values are demonstrated in figure 10. When mean control (n=12) and MMC treated (n=12) values were compared, control blebs were found to be significantly thicker (p

< 0.000004; Student *t* test) than MMC treated eyes. This trend was also observed when comparing dogs within any given treatment group ($p < 0.002$). Conversely, when actual measurements were used to compare capsule thickness (control vs. MMC treated eyes) within any given individual, 3/12 dogs (1, 8, and 9) failed to show a significant difference ($p < 0.05$) between control and treatment eyes. This is depicted in figure 10 by the overlapping of the standard error of the mean bars generated by wide variations in obtained measurements. The gross relationship of the globe with its accompanying filtration bleb is depicted in figure 11. Partial collapse of the globe was artifactually created when a calotte of the filtration bleb was removed for future *in vitro* fibroblast culture studies. Qualitative differences were also observed between the capsules of MMC treated and control eyes. Figures 12 and 13 demonstrate the histologic appearance of a segment of capsule wall from a control and MMC treated eye. Trichrome staining was utilized to accent the regional fibroblasts and associated collagen fibers. Comparison of these figures revealed the capsular wall of the MMC treated eye to be notably thinner than those of control eyes, with a more loose arrangement of collagen fibers. In all eyes, the capsule was limited to the immediate perimeter of the scleral explant without anterior extension toward the limbus. Therefore, the clinically larger filtration bleb observed in most MMC treated eyes (fig. 2) relative to control eyes (fig. 1), could not be explained by an increase in capsular wall thickness or internal lumen diameter.

Cell cultures from explanted tissues

Figure 14 depicts initial fibroblast outgrowth from harvested tissue sections placed in tissue culture flasks. Although tissues were collected from all 12 dogs, bacterial contamination of the fetal calf serum used in the first group (dogs 9 - 12; 1 month postoperative group) reduced the available sample size to 8 dogs (2 and 3 months postoperative groups). Mean cell counts from control eyes were found to be significantly greater ($p<0.0001$) than MMC treated eyes when comparing all dogs ($n = 8$), or dogs within a given treatment group ($n = 4$). Figure 15 depicts the more prolific growth of control cells relative to MMC treated cells.

Discussion

Numerous clinical studies support the efficacy of a single perioperative treatment of MMC in glaucoma patients undergoing filtration procedures.[17,19,20,28] When tissue levels of MMC were measured following a single intraoperative treatment, values fell well below the minimum antiproliferative level within a few days.[29] Half-lives of MMC and 5FU have both been shown to range from 0.18 to 0.45 hours depending on the periocular tissue sampled.[29] From this it follows that MMC's reported *in vitro* antiproliferative activity lasts much longer in the clinical situation than the tissue concentrations would suggest. One mystery of single dose antimetabolite therapy is its apparent ability to retard fibroblastic proliferation over an extended time interval. Tahery and Lee[30] have hypothesized that natural alkaloids such as mitomycin may, even with short exposure times, have the ability to "suppress cellular proliferation completely" since their activity is relatively cell cycle non-specific when compared with 5FU. High concentrations of MMC may result in a cytocidal effect in addition to inhibiting fibroblast proliferation.[21]

Clinical examination of the 12 MMC treated eyes in this study failed to demonstrate any of the previously reported signs of epithelial toxicity (indolent ulceration, striate melanokeratosis, or superficial punctate keratitis and filament formation) commonly associated with serial 5FU therapy.[20,31-36] In fact, the authors were only able to identify one report where transient superficial corneal vascularization was observed anterior to the filtration bleb in rabbits undergoing filtration surgery with concurrent MMC therapy.[19] This apparent lack of local toxicity following MMC therapy appears to be more related to the selected treatment protocol than to the pharmacologic properties of the particular antimetabolite. Serial topical treatment using MMC in the management of pterygia has been associated with conjunctival irritation, excess lacrimation and mild superficial punctate keratitis.[37]

Use of glaucoma drainage devices in man has traditionally been reserved for those individuals who have not been successfully managed using conventional medical therapy or filtration procedures. This practice has evolved due to a high incidence of postoperative complications. Included are transient hyphema, collapsed

anterior chambers with or without choroidal detachment, tube touch to the lens, iris or cornea, tube block with iris or vitreous, tube migration and cataract formation.[7,38-43] Additional, albeit rare, complications include endophthalmitis,[44-46] persistent cystoid macular edema[47] and hypertropia secondary to bleb encapsulation.[48,49] Despite these occurrences, the reported overall success rate of seton surgeries range from 50-96%.[42,50]

The present authors have previously reported a high incidence of implant extrusion with accompanying tube migration when scleral explants were positioned immediately below the dorsal bulbar conjunctiva.[9] It is believed that a return to the more conventional subtenon's location in this study is responsible for the absence of these clinical complications. Collapse of the anterior chamber with resultant dyscoria and corneal endothelial contact with tube or iris was the most frequent complication in this study; a finding more prevalent in MMC treated eyes. Surgical intervention was not performed in order to avoid further influences on the development of the associated filtration bleb. All anterior chambers were reformed following an average of 10.8 days. Associated intraocular complications included tube induced dyscoria and focal non-progressive corneal edema. Similar findings have been recorded in human clinical trials.[51] Tube tip incarceration within the iridal stroma did not appear to significantly impair the operation of the drainage device as low IOP's were maintained and filtration bleb characteristics were preserved. Tube block by the iris, due either to poor placement or shallowing of the anterior chamber, occurs in 5-11% of human cases.[42] Management can be through laser or surgical intervention. Nd:YAG laser has a poor success rate for unblocking the orifice, largely because of the difficulty in directly focusing energy on occluding tissue that has been sucked inside the tube lumen. Alternatively, a small surgical entry tract allows the incarcerated iris to be swept out of the tube.[42] It was observed in this clinical trial that all tube tips ultimately became free from the adjacent iris stroma. One individual from the present study developed a linear subcapsular cataract in its MMC treated eye. In human patients there is strong evidence that glaucoma surgery is cataractogenic. Reports suggest that approximately 1/3 of eyes which develop cataracts go on to result in a reduced visual acuity of at least 2 lines from the preoperative baseline as measured using the Snellen chart.[43] Associated risk factors in man include shallow or flat anterior chambers, preexisting lens opacity, steroid use, uveitis, miotic therapy and

lens trauma.[43] The role of MMC in cataract formation remains unknown at this time. Also unclear is the effect of antimetabolites on uveal tissues. Although some authors suggest that 5FU may have a beneficial effect on intraocular inflammation,[35] others have reported an acute granulomatous iritis following 5FU therapy for a failed trabeculectomy.[52] One study measured aqueous flare in 16 glaucomatous eyes which had undergone trabeculectomy surgery with concurrent 5FU or MMC therapy. Following conversion of flare values to albumin concentration (mg/dl), it was determined that the 5FU group had significantly higher values than those found in the MMC group.[53] Furthermore, when MMC is administered only once postoperatively, it becomes undetectable in the aqueous humor within 6hr of administration.[29] Although these reports would tend to dismiss concerns of local irritation of uveal tissue from single dose MMC, one eye from the present study did show a mild suppurative anterior vitritis of unknown etiology. While not available at this facility, fluorophotometery could have been used to better address this issue. In man, factors associated with increased risk of endophthalmitis include increased axial length, thin and leaky bleb, conjunctivitis, upper respiratory infection, and the winter season.[54] Similar associations have not been documented in the veterinary literature.

Filtration Blebs

Central to any glaucoma filtration or seton surgery is the integrity of the associated filtration bleb. Following implantation of a glaucoma drainage device, proliferation and migration of a relatively limited local population of conjunctival and scleral fibroblasts, without additional repopulation from vascular sources as was previously believed, combine to form a fibrous capsule surrounding the scleral footplate.[25] Once formed, this interface delays uptake of egressing aqueous humor into the regional vascular channels, and in so doing regulates IOP. The two most widely reported causes of bleb failure following filtration surgery are bleb scarring and bleb encapsulation (BE).[1,21,55] In veterinary ophthalmology, bleb encapsulation has been reported following glaucoma implant surgery[9] while scarring of the filtration site remains the expected outcome for most canine glaucoma filtration procedures.

Although the cause of BE is not known, four basic tenants exist. 1) Non-contractile, collagen-producing fibroblasts appear primarily responsible for the process of BE, while wound healing following filtering surgery is dependent on contractile fibroblasts. 2) 5FU has a more potent toxic effect on the process of wound healing than on bleb encapsulation. 3) Collagen-producing fibroblasts may be less sensitive that contractile fibroblasts to the destructive effects of 5FU. 4) Inflammatory mediators are important triggers of bleb encapsulation.[56]

Encapsulated blebs typically form between the second and eighth week after surgery.[55-57] After surgical trauma, inflammation at the wound site occurs. This is followed by migration and proliferation of local fibroblasts with resultant production of collagen, elastin and mucopolysaccharides. Two subsets of fibroblasts exist, one specifically programmed to synthesize collagen and the other destined to produce contractile proteins and become myofibroblasts.[58] This fibroblast-myofibroblast transformation is promoted by several factors including tissue hypoxia, wound tension, and tension in the surrounding tissues.[59] It has been further suggested that a subpopulation of human T-lymphocytes (Thy-1.2+, L3T4-, Lyt2-) normally stimulate such fibroblast activation.[60] Flattening of the bleb results from myofibroblast contraction.[58] From these findings it has been suggested that non-contractile collagen-producing fibroblasts play the major role in the process of BE, while contractile fibroblasts represent the primary cell type responsible for wound healing following filtration surgery.[56]

The clinical appearance of a bleb does not always reflect its function.[55] Nevertheless, glaucoma filtration blebs that are working well tend to be low and diffuse, covering at least one quadrant of the globe without signs of marked distention.[55] Those which result from glaucoma seton surgeries approximate the associated scleral explant. They often have a watery, succulent, translucent appearance and are relatively avascular. The most useful clinical sign of good bleb function is the development of fine, confluent microcysts within the conjunctival epithelium suggesting movement of aqueous into the subconjunctival space.[1,55,61] This process typically takes about 1 month to occur in human patients.[57] Encapsulated blebs often appear as sharply

defined, distended domes overlying the filtration site, with generalized engorgement of the subconjunctival tissue.[1,55]

Clinical evaluation of the filtration blebs of MMC treated eyes from this study demonstrated a trend towards fuller and less vascular blebs with an increased incidence of conjunctival microcysts when compared to control eyes. Bleb encapsulation was not observed. In human glaucoma patients, elevations in serum ALT[62] as well as prior conjunctival surgery have been associated with an increased incidence of BE. Conversely, a reduced incidence of postoperative BE has been observed in women (especially under 50 yrs) and in patients undergoing surgery within 2 years of the diagnosis of glaucoma.[57] When encountered in human patients, the majority of individuals are successfully managed through the use of anti-glaucoma medication, digital massage and contact lens application. Rare individuals will require surgical intervention in the form of argon laser therapy,[63,64] needling,[57] or total resection[57] of the capsule. Despite these temporary complications, the long-term prognosis for these patients is generally favorable. Unlike BE, scarring of the filtration site with flattening of the filtration bleb is irreversible. Reduction of the bleb contour, while absent in all MMC treated eyes, was observed in 9/12 control eyes in the present study. Similar results have been reported in human and rabbit patients undergoing filtration procedures in combination with 5FU[15,56,65,66] or MMC[17,29,66] therapy. Also observed in 2/12 MMC treated eyes from our study was a dorsal bulbar conjunctiva which was sufficiently loose so as to overhang the adjacent cornea. Although no attempt was made to correct these individuals, surgical excision of the excess with reapposition is the treatment of choice in man.[64] In such cases the anterior chamber is not lost as the overhanging bleb is usually nonfunctional.[64] One complication which has been widely reported following antimetabolite therapy is leakage from excessively thin-walled blebs.[64] This often results in clinical endophthalmitis with the patient having a very poor long-term visual outcome.[64] Other than interspecific susceptibility differences, two factors may be responsible for its absence in the present study. Use of a two-layer closure, as was utilized in the current study, has significantly reduced the reported occurrence of this complication.[67] It has been noted that blebs most commonly leak late in the postoperative period, sometimes many years after the filtration surgery, as blebs continue to thin.[55,68] Dogs in this study were

followed for a maximum of 3 months. It is conceivable that a longer follow-up period could have increased the incidence of this complication in the present study.

When assessing the effects of MMC therapy on glaucoma surgical procedures, an understanding of the regional pharmacokinetics is required. The actual amount or concentration of MMC being delivered to the subconjunctival tissues with the current techniques is unknown.[20] Authors have measured MMC concentrations in ocular tissues using high-performance liquid chromatography. Mitomycin C has been shown to be rapidly cleared from the conjunctiva, sclera and aqueous humor of rabbits following a single injection, with a reported half-life ranging from 0.18 to 0.45 hr.[29] In the same study it was found that irrigation with 200 ml of saline further reduced the concentration to 1/5th in the sclera and 1/15th in the conjunctiva without changing the half-life. It would follow that MMC's reportedly favorable clinical antiproliferative activities last much longer in the clinical setting than the tissue concentrations would suggest. Also of interest is the interactive role of corticosteroids on filtration bleb encapsulation. The role of adjunct anti-inflammatory therapy, particularly systemic corticosteroids, in controlling postoperative fibrosis following Molteno implant surgery remains controversial.[49] Cell culture data has demonstrated that glucocorticoids have a biphasic effect, supporting fibroblast growth until quite high doses are maintained, at which time inhibition takes over.[69] The currently recommend protocol, a protocol which was followed in this clinical trial, is that of short-term high dose corticosteroid therapy to address the immediate postoperative inflammatory reaction followed by rapid discontinuation of topical steroids to reduce the chance of BE.[69]

Histologic examination of filtration blebs from the present study revealed significantly thinner fibrous capsules with a more loosely arranged collagen and fibroblasts matrix in the MMC treated eyes. Similar findings have previously been reported in rabbits[19] and humans[70] following combination glaucoma filtering surgery and MMC therapy. Clinical reviews comparing the pressure lowering effects of 5FU and MMC when combined with glaucoma filtering procedures demonstrated superior results using MMC therapy.[17,20] Other authors have reported that this reduction in IOP is the result of antimetabolite induced structural alterations to the filtration bleb.[15] Lending credence to this theory is the apparent lack of MMC induced histologic alterations

of the ciliary body processes which would be suggestive of decreased aqueous humor production.[21] Histologic examination of the eyes from the present study also revealed normal anterior uveal structures. Use of tonography or fluorophotometry has been recommended to further verify the mechanism of decreased IOP.[15] Although fluorophotometry was not available for the present study, tonography was available and performed. Statistical analysis failed to demonstrate a significant difference between tonographic values obtained from control and MMC treated eyes. This differs from an earlier report where rabbits treated with 5FU labeled bioerodible polymers demonstrated greater coefficient of outflow values (C = 0.53 ul/min/mmHg) versus control eyes (0.29 ul/min/mmHg).[71] It is the authors' opinion that the extremely low resting IOP's observed in selected eyes from the present study prevented them from achieving the normally observed compensatory rise in IOP following challenge with a 10gm weight. This resulted in an erroneously low coefficient of outflow values when calculated using human tonographic tables.

Finally, the long-term effects of MMC on subconjunctival fibroblasts replication and outgrowth was evaluated by comparing fibroblast growth from control and MMC treated eyes upon completion of the *in vivo* component of this study. Results confirmed a continued inhibitory effect of MMC on harvested subconjunctival fibroblasts when compared to cells derived from control eyes. Earlier *in vitro* studies by this author[26] and others[22] demonstrated the inhibitory effects of both 5FU and MMC on the growth of subconjunctival fibroblasts. However, in one of these studies,[22] the author commented that the long-term effects appeared less marked in tissue samples taken after experimental filtering surgery than was observed during previous *in vitro* tissues culture studies. Such differences may be pharmacokinetic in origin as drug delivery and penetration in a cell monolayer would be expected to be greater and with a more marked effect than that delivered by a sponge *in vivo* where retarded penetration through the subconjunctival and scleral tissues during the 5 minute exposure period would be anticipated.[22,23] Quantitative differences between *in vitro* and *in vivo* components of the present study were not examined.

Addendum

Since the inception of this study, the authors have had the opportunity to perform the drainage implantation surgery with concurrent MMC therapy on four clinical cases. Of these, 3/4 were in normotensive globes with narrowed drainage angles and buphthalmic fellow eyes while 1/4 was a hypertensive globe referred due to lack of response to medical therapy. All dogs were treated for 5 minutes perioperatively with a reduced volume (0.25ml) of MMC. Follow-up times range from 9-16 months. To date all eyes are visual, possess a smooth, uniformly distended filtration bleb overlying the scleral explant, and have maintained an IOP of 8-12 mmHg. None have demonstrated any of the complications encountered in the present study. The author suggests that those intraocular complications described in this report may result from an excessive MMC dosage, and that this newer protocol may continue to offer the desired therapeutic response without the associated intraocular complications.

Table 1 Preoperative and postoperative medications administered during the *in vivo* clinical trial. No medications were administered beyond postoperative week four.

Medication	**Interval of Treatment**				
	48hrs Preoperatively	**Days 1-7 Postoperatively**	**1-2 Weeks Postoperatively**	**2-3 Weeks Postoperatively**	**3-4 Weeks Postoperatively**
Gentamicin sulfate (topical) (OU)	q8hr	q8hr	q12hr	q12hr	q24hr
Dexamethasone phosphate (0.1%, topical) (OU)	q8hr	q8hr	q12hr	q12hr	q24hr
Prednisone (0.75 mg/kg PO)	q12hr	q12hr	q24hr		
Amoxicillin (10mg/kg PO)	q12hr	q12hr (until day 5)			

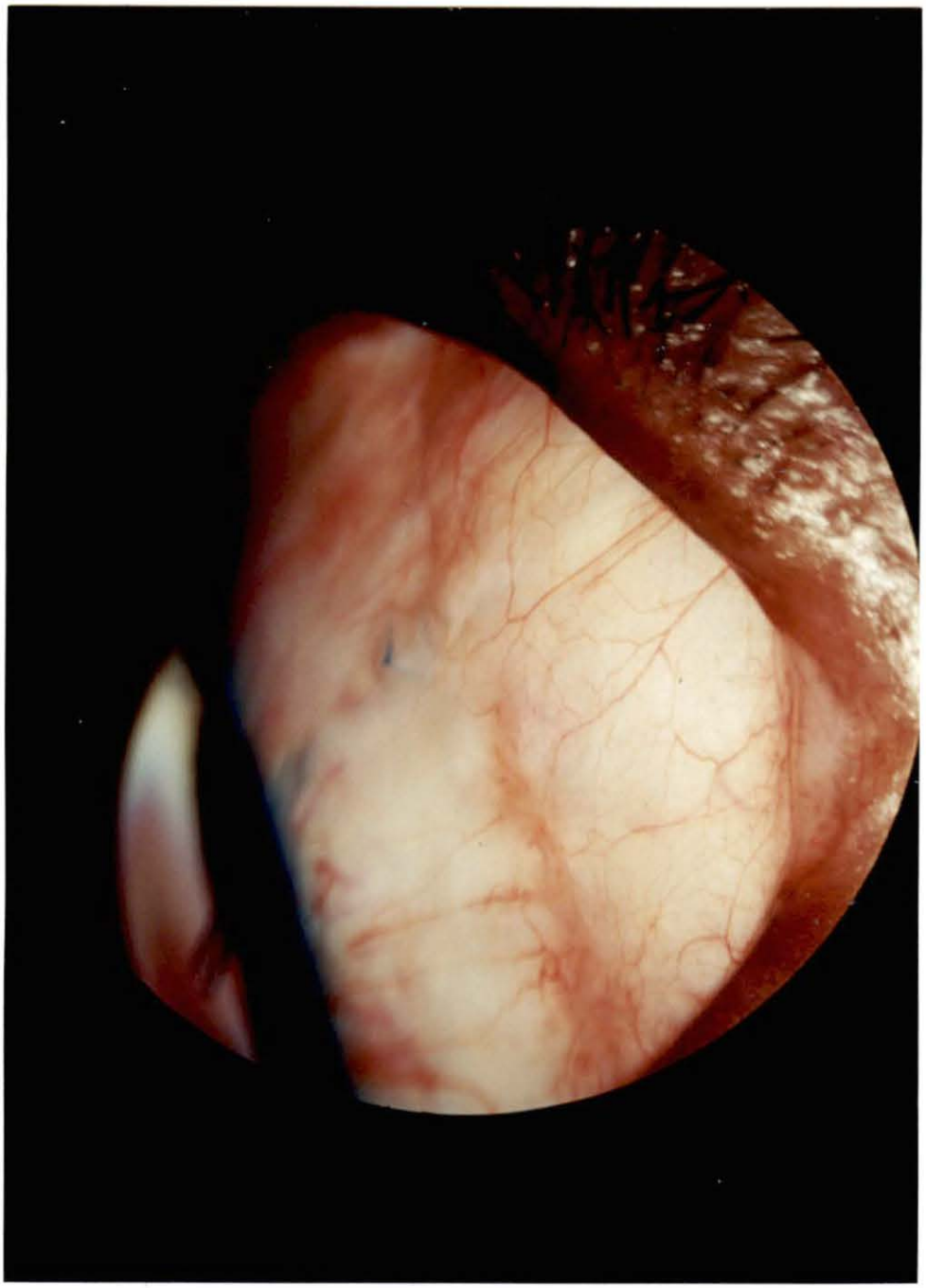

Fig. 1 The clinical appearance of the filtration bleb from the control eye (OS) of dog #1 on postoperative day 93 as viewed from the lateral palpebral commissure. The bleb was tightly adhered to the underlying sclera anterior to the site of previous conjunctival incision. Caudally, a moderate sized smooth distention overlay the scleral explant of the drainage device. The entire conjunctiva was uniformly vascularized. In both eyes the subconjuctival region of the drainage tube and its fixation suture were easily seen. This represented a normal variation in thickness of the overlying tissues. There was no evidence of wound dehiscence or leakage.

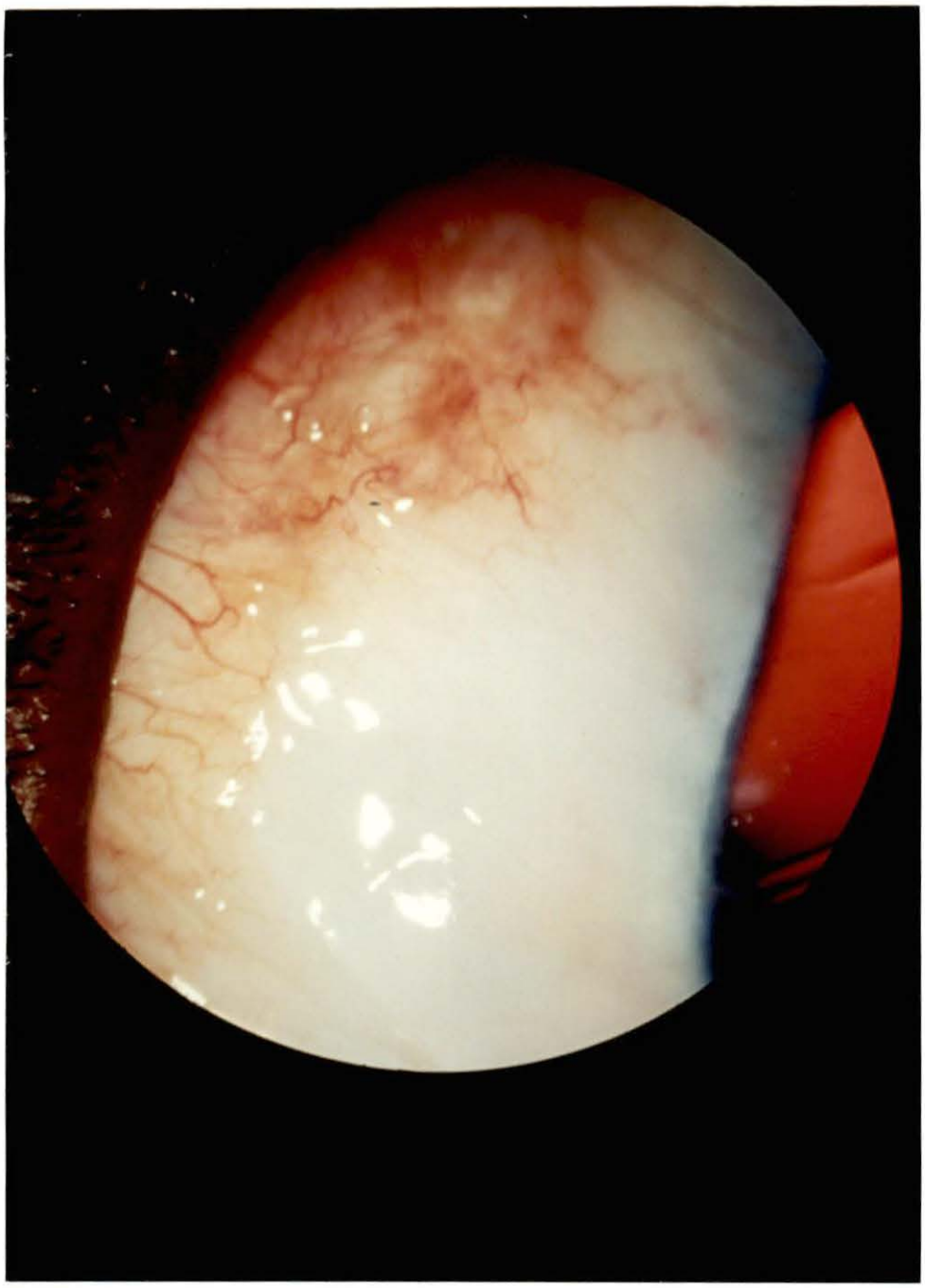

Fig. 2 The clinical appearance of the filtration bleb from the MMC treated eye (OD) of dog #1 28 days postoperatively as viewed dorsolaterally. The bleb was uniformly distended anteriorly to the limbus. This was in sharp contrast to that seen in the fellow control eye (fig. 1). Also apparent was a marked pallor of the overlying anterior bulbar conjunctiva which was readily differentiated from the uniform vascular pattern found in the control eye (fig. 1). Close examination revealed intermittent conjunctival microcysts along the length of the healed incision. These cysts were observed in 3/12 MMC treated eyes and 1/12 control eyes.

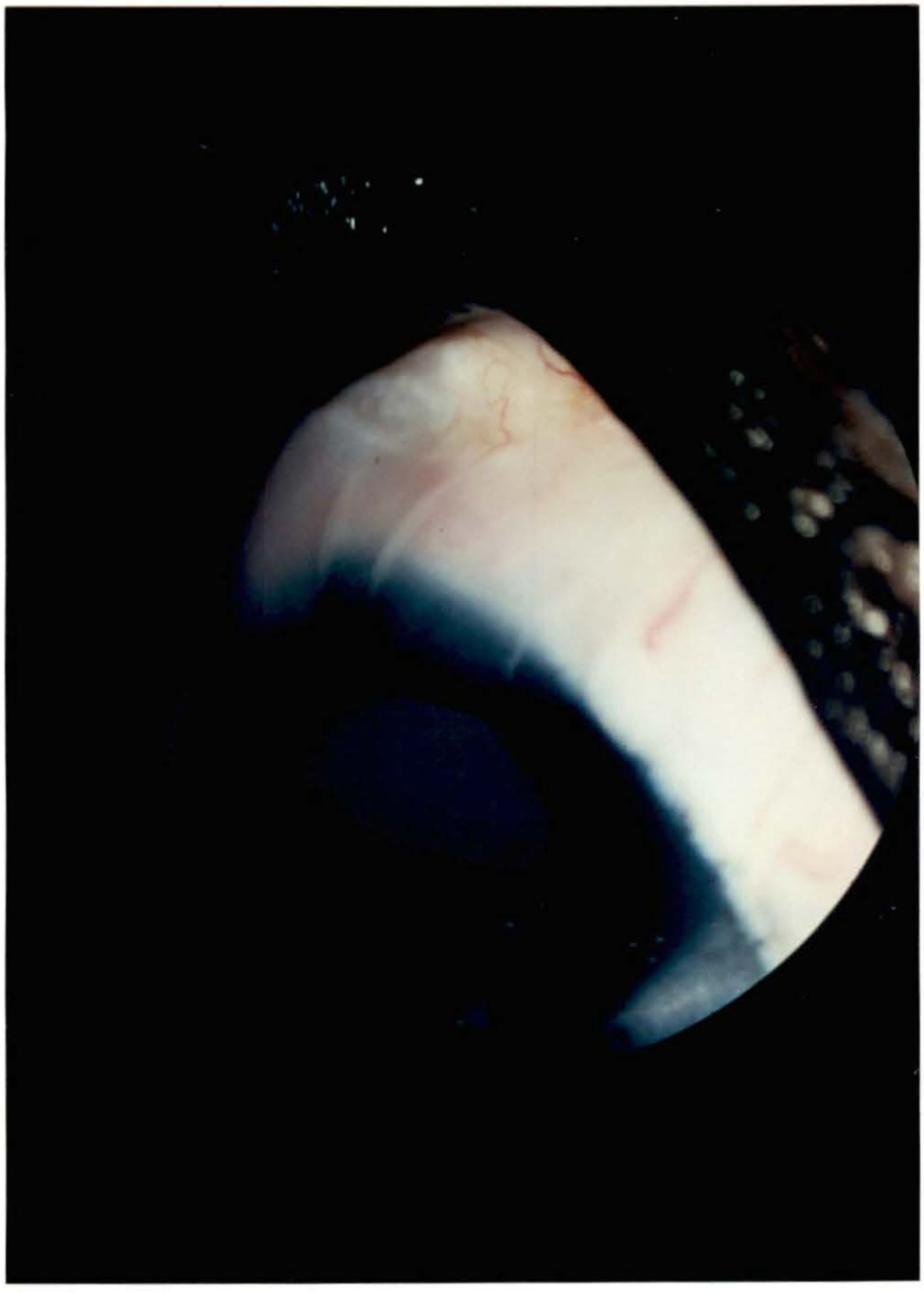

Fig. 3 This clinical photograph demonstrates the abnormal mobility of the dorsal bulbar conjunctiva which was identified in 2/12 MMC treated eyes. In this individual a pendulous dorsal bulbar conjunctiva was observed to overhang the adjacent cornea (OS). This effect was most apparent during upgaze of the affected globe.

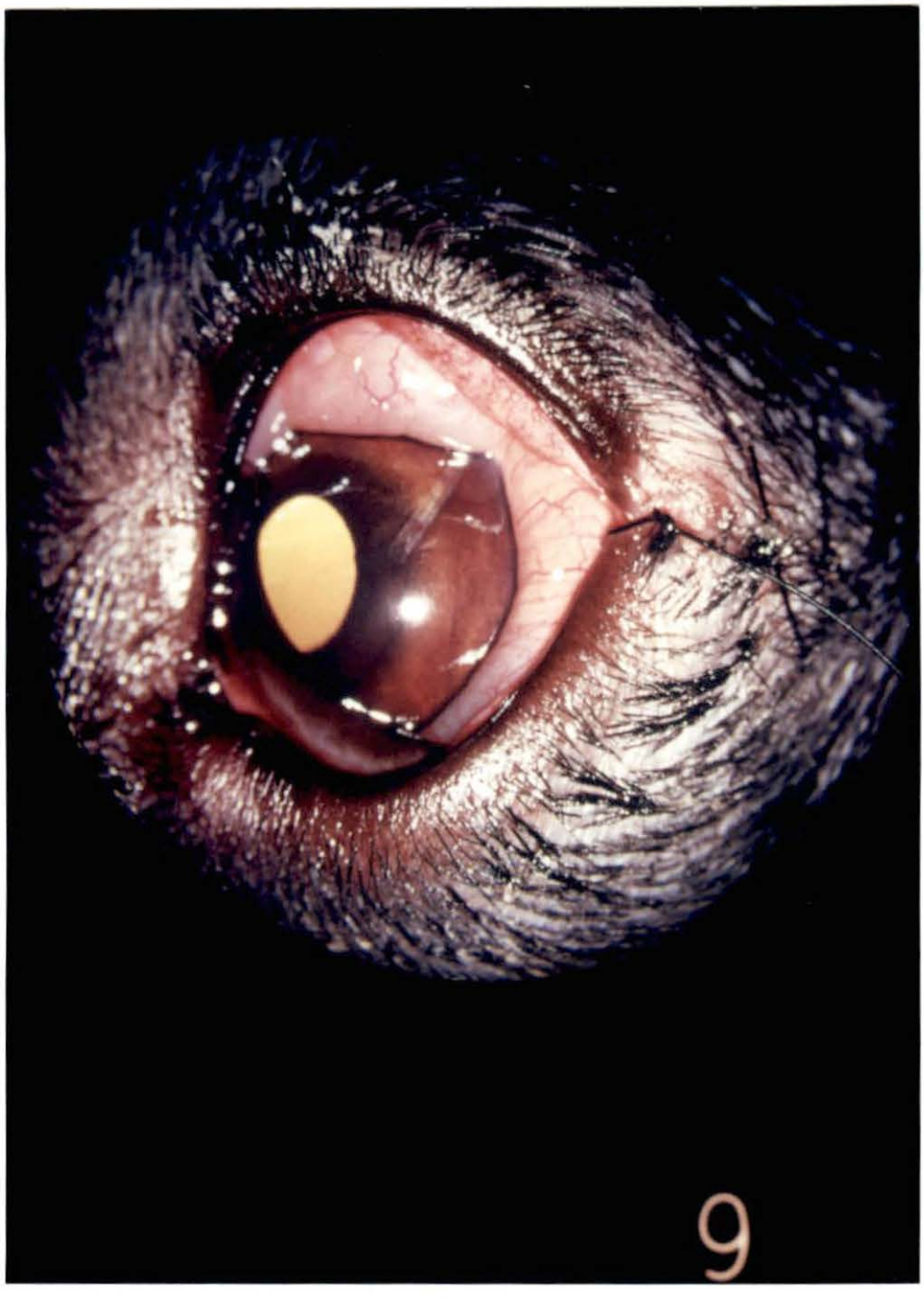

Fig. 4 This photograph shows the clinical appearance of a left eye following sudden spontaneous evacuation of fluid from its anterior chamber. The surrounding conjunctiva is markedly distended and mildly hyperemic. Without aspirating it was impossible to determine if the swelling was a cystic distention of the bleb or subconjunctival edema. Note the small amount of organized blood located within the drainage tube. All eyes regained normal anterior chamber depth without surgical intervention.

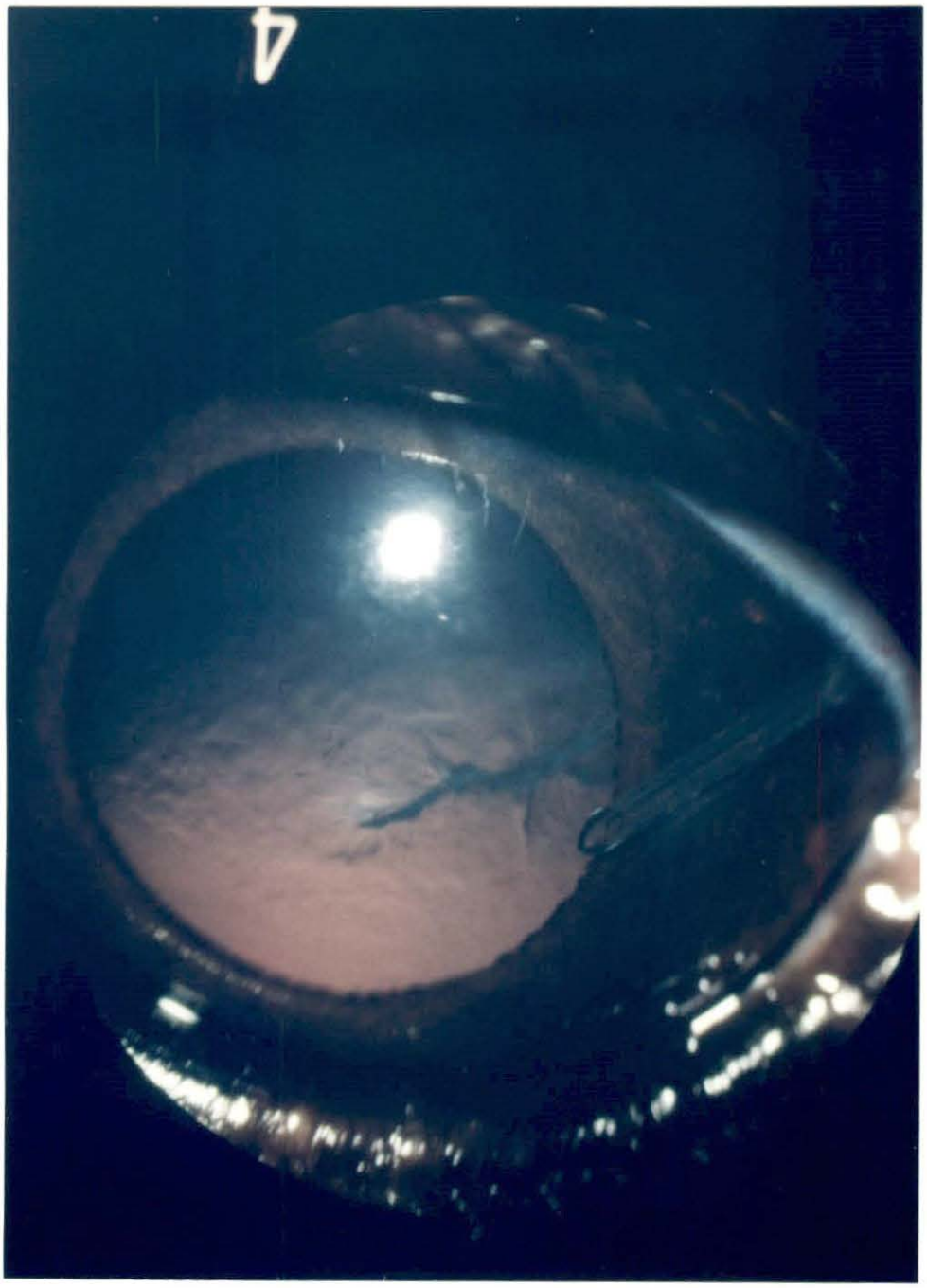

Fig. 5 This figure of a MMC treated eye (OS) shows a linear branching anterior subcapsular cataract with orientation parallel to the implanted drainage tube. Contact between the tube and lens was not observed. Although initially recognized on postoperative day 31, there was only slight advancement of the lesion at the time of this photograph (day 95).

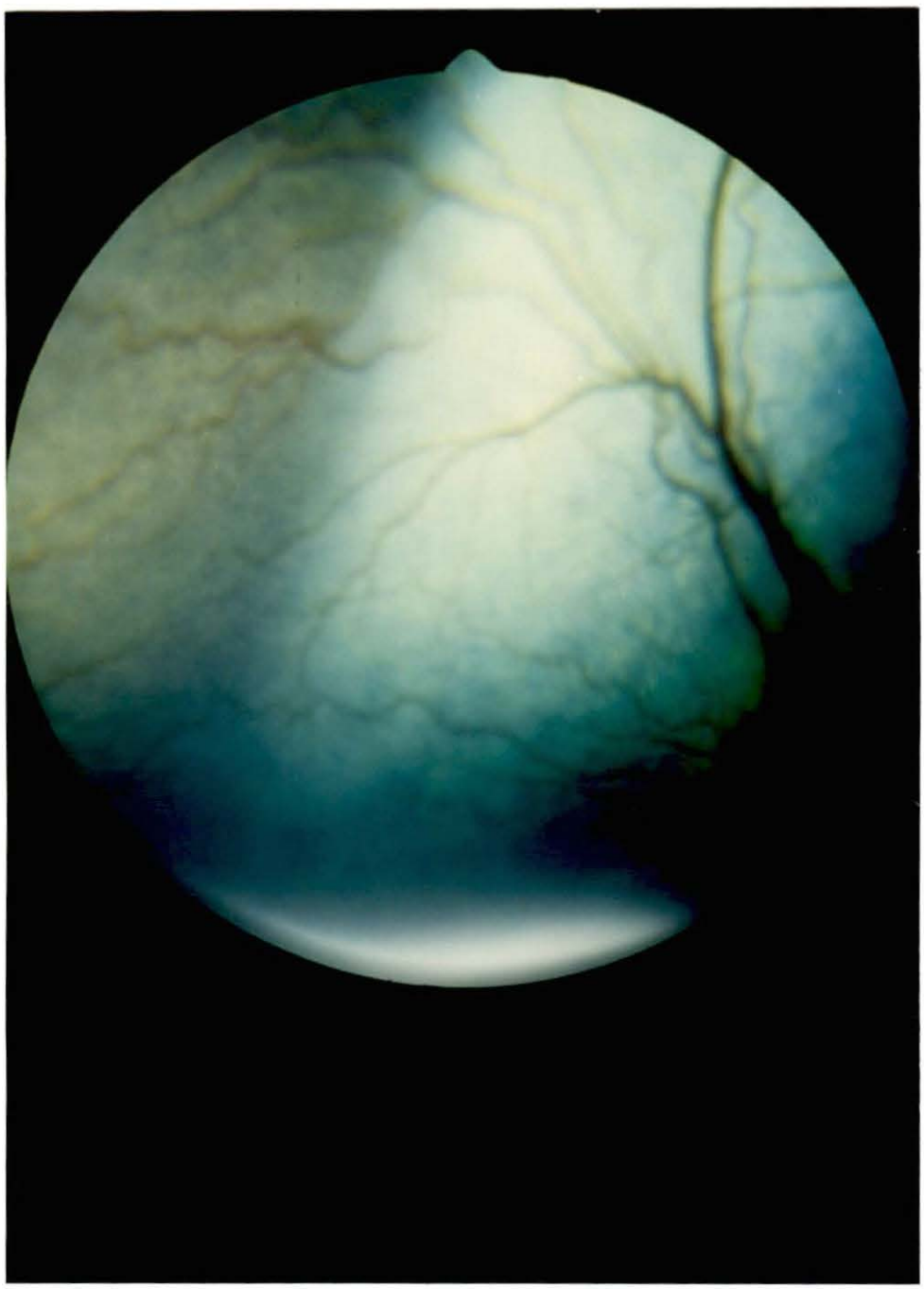

Fig. 6 Photograph of the right fundus of dog #4 on postoperative day 63. A large linearly defined hyporeflective area, similar to a bullous retinal detachment, was seen to extend obliquely across the dorsolateral tapetal fundus of this and all other eyes. The involved area outlined the boundaries of the affixed scleral explant. Subsequent histologic examination showed normal apposition of the neurosensory retina.

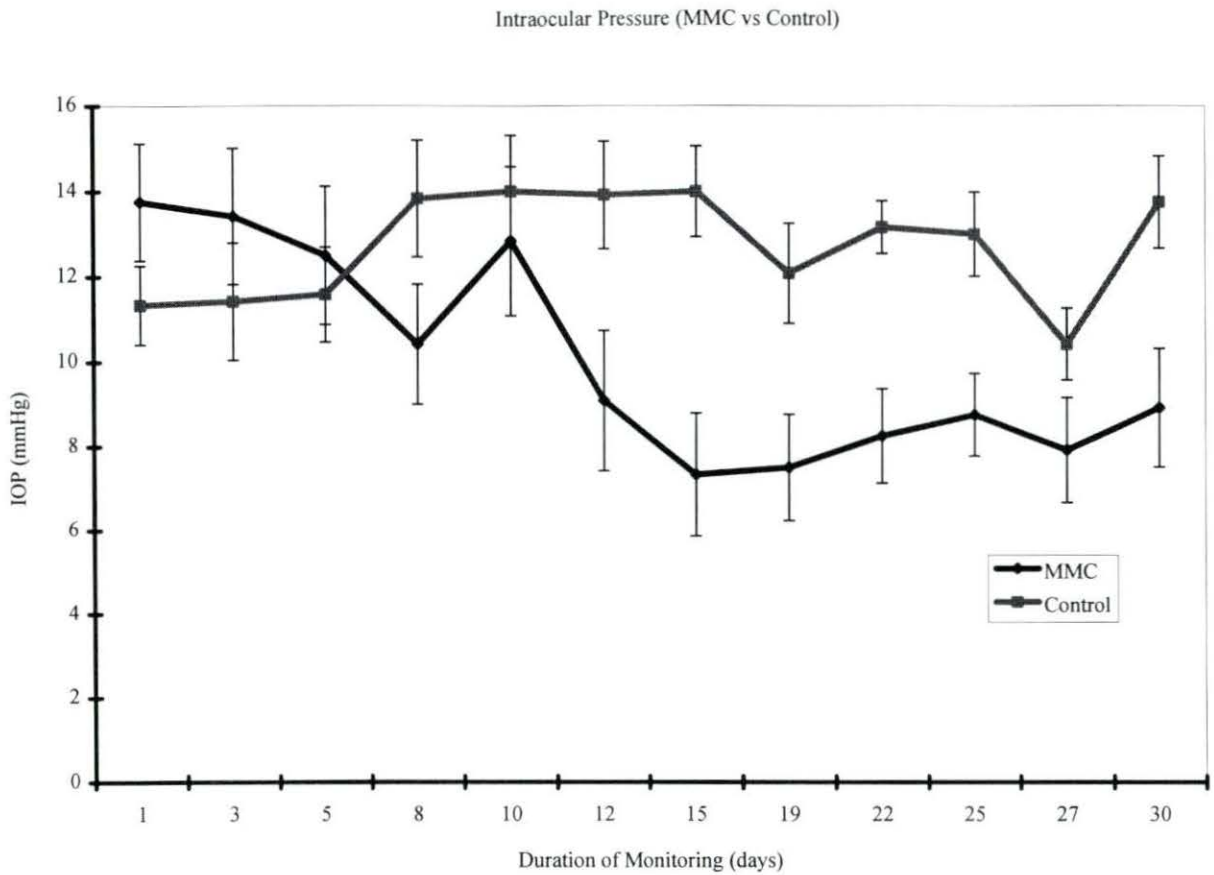

Fig. 7 This chart compares the mean IOP (mmHg) of all dogs being monitored up to postoperative day 30 (n = 12). Values from MMC treated eyes were significantly lower ($p < 0.01$; Student *t* test) than those obtained from fellow (control) eyes. Vertical bars represent the standard error of each mean.

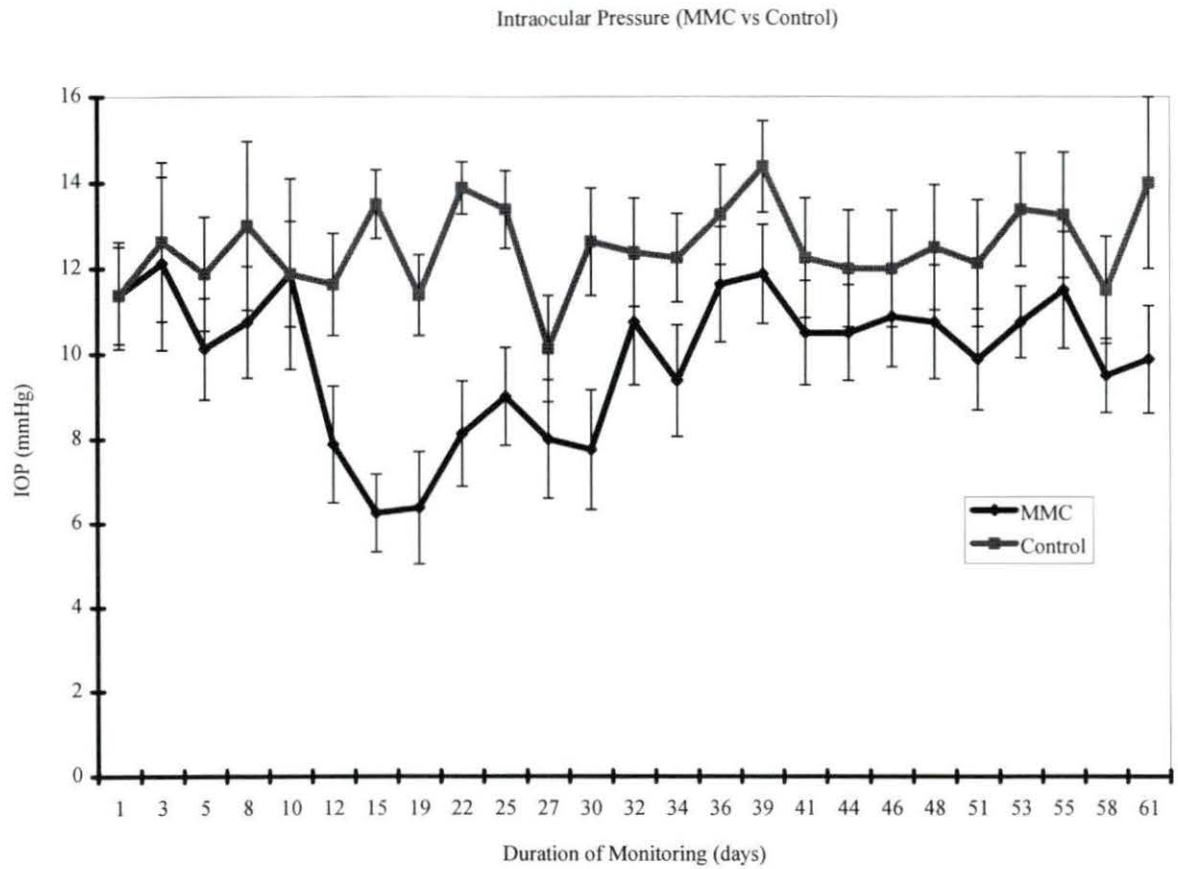

Fig. 8 This chart compares the mean IOP (mmHg) of all dogs being monitored up to postoperative day 61 (n = 8). Values from MMC treated eyes were significantly lower ($p < 0.01$; Student *t* test) than values obtained from fellow (control) eyes. Vertical bars represent the standard error of each mean.

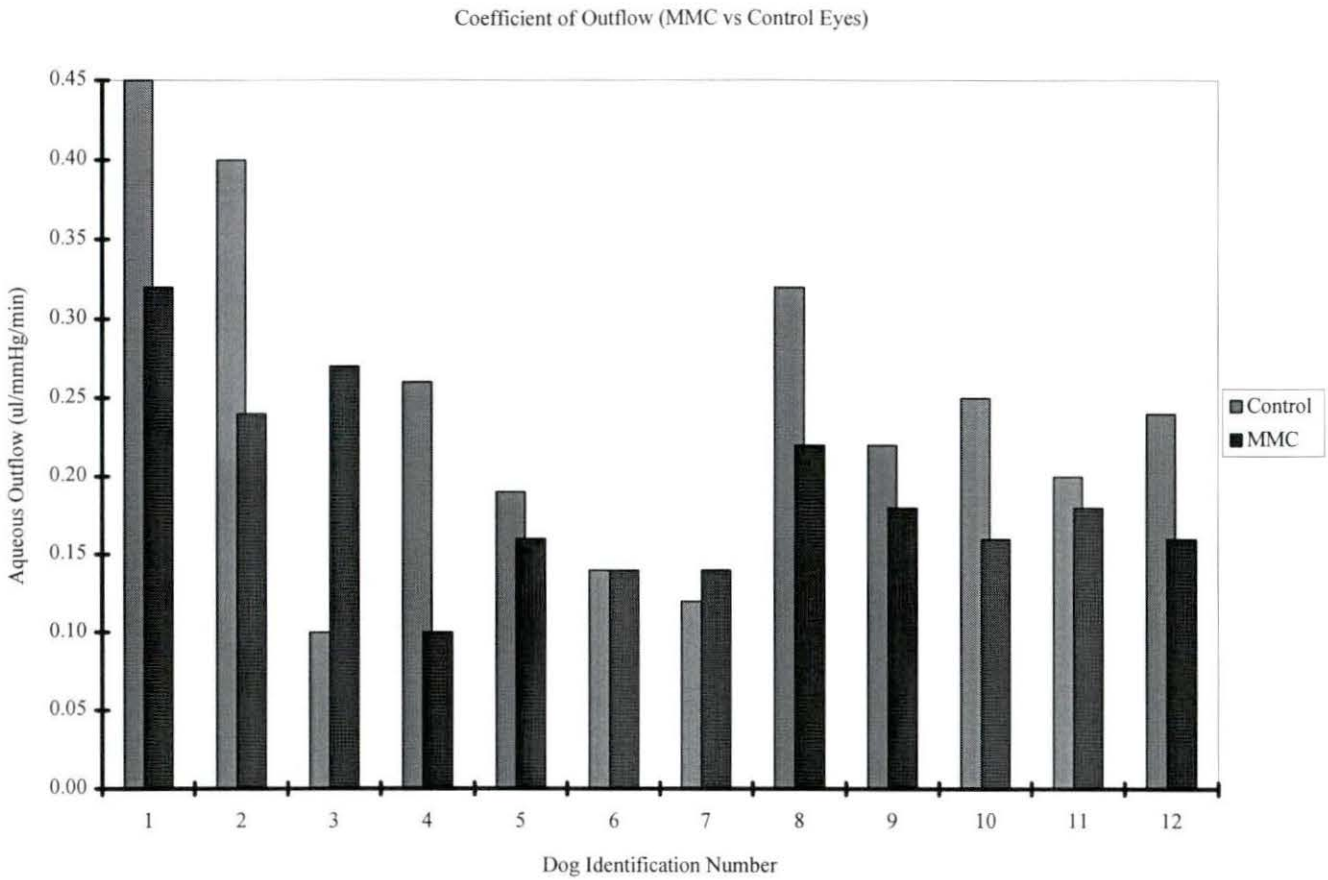

Fig. 9 Tonographically derived "C" values (ul/mmHg/min) obtained from both MMC treated and fellow control eyes are compared. Although 9/12 individuals showed a trend towards higher values from control eyes than from MMC treated eyes (dogs 3,6, and 7 excluded), this trend was not significant ($p<0.05$) when comparing all dogs (n=12), or dogs within any particular treatment group (n=4) using the Student *t* test.

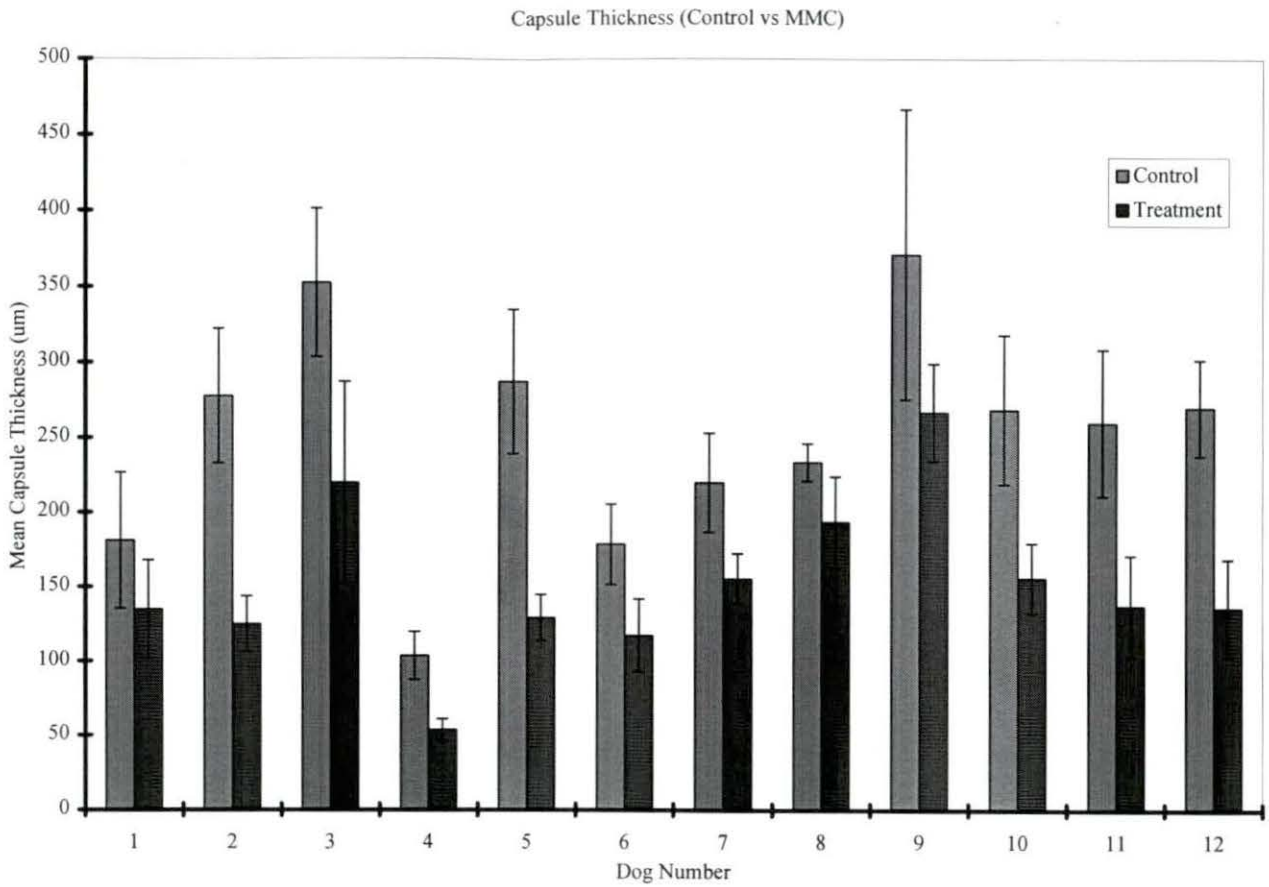

Fig. 10 This graph displays the mean capsular thickness (μm) of control and MMC treated eyes for all dogs (n = 12). Vertical bars at the peak of each histogram represent the standard error of each mean. MMC treated eyes had significantly thinner capsules when comparisons included all dogs ($p<0.000004$) or dogs within individual treatment groups (n = 4) ($p<0.002$). When comparing values between control and MMC treated eyes within a given individual, dogs 1, 8 and 9 failed to show significant ($p< 0.05$) capsular thinning of MMC treated eyes relative to control eyes.

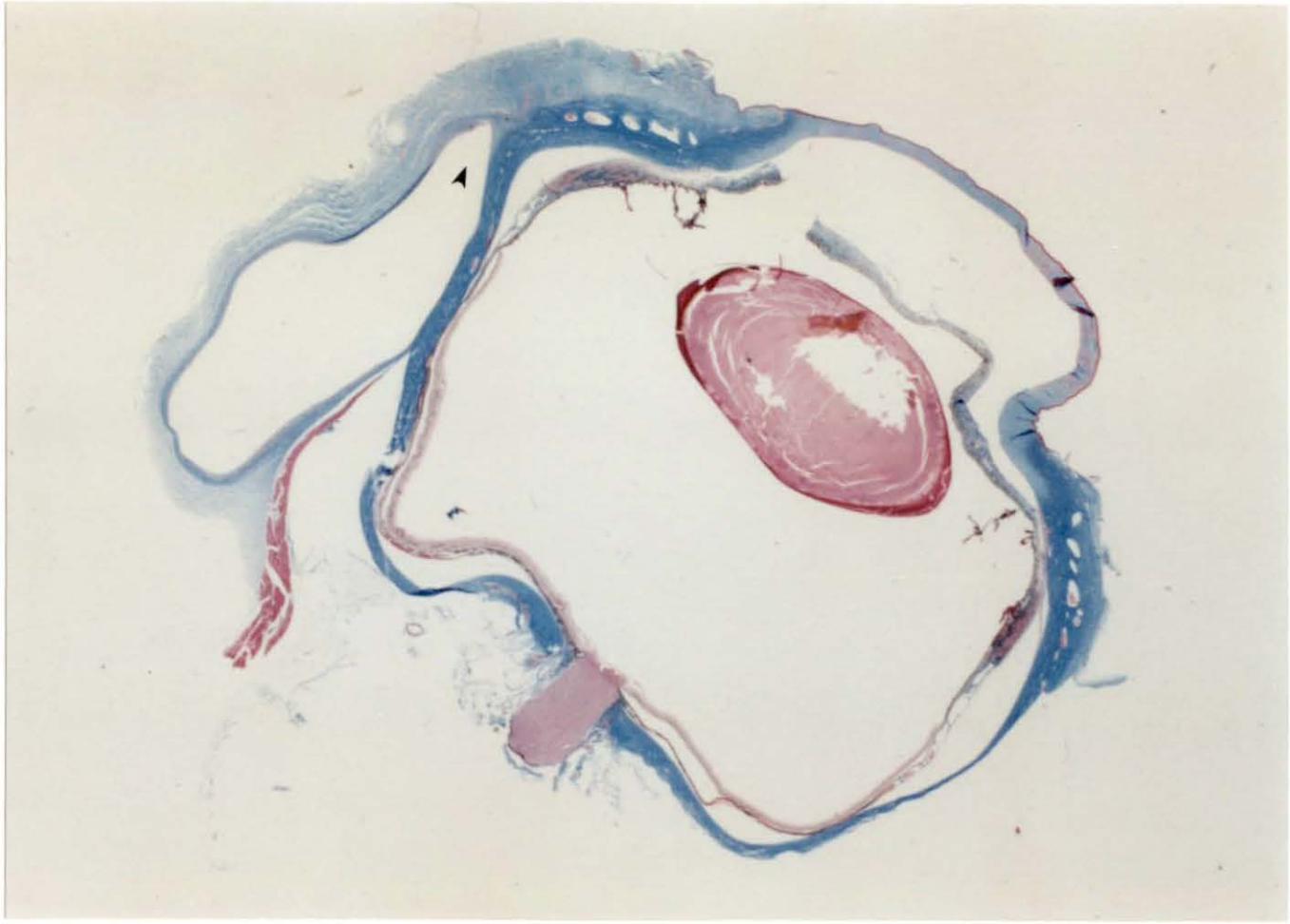

Fig. 11 The gross relationship of the globe with its accompanying filtration bleb is depicted in this photomicrograph. Partial collapse of the globe was artifactually created when a calotte of the capsule was removed for future *in vitro* fibroblast culture studies. Arrowhead identifies the anterior extent of pocket (Trichrome x6)

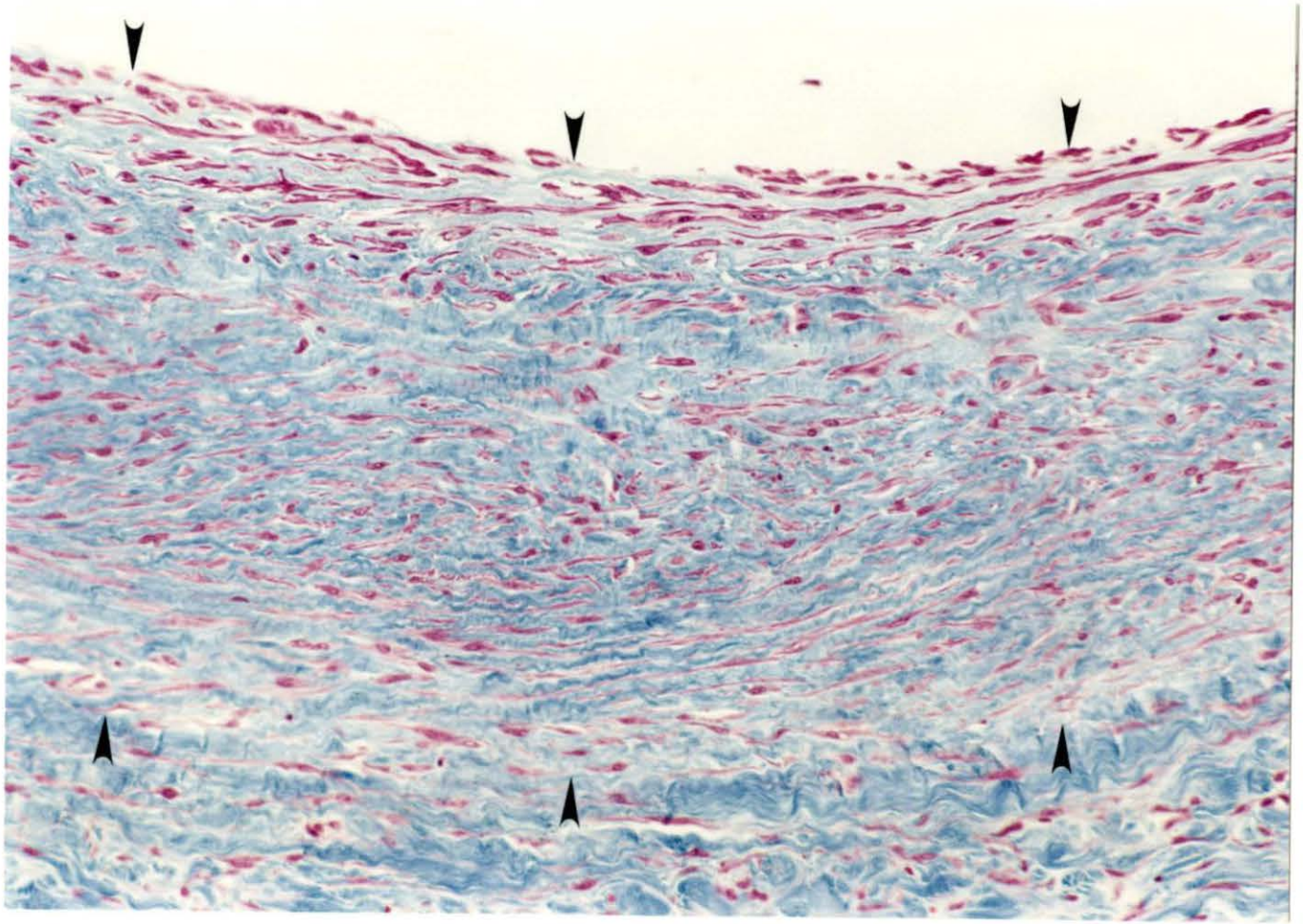

Fig.12 This photomicrograph reveals the histologic appearance of the filtration bleb of a control eye. Note the thick capsular wall with tightly arranged collagen fibers and numerous fibroblasts. Arrowheads depict capsular thickness. (Trichrome stain x160)

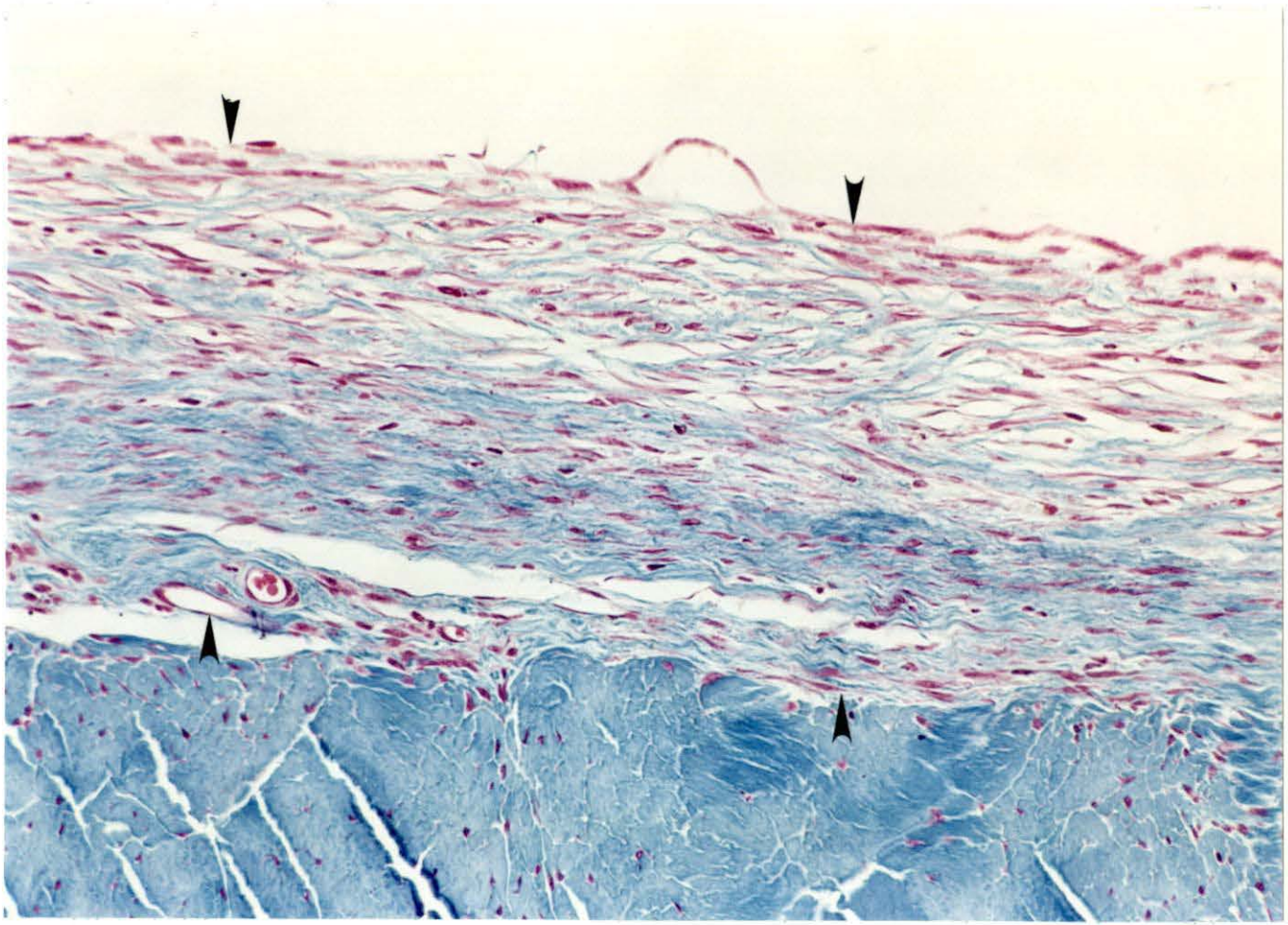

Fig. 13 This photomicrograph reveals the histologic appearance of the filtration bleb of a MMC treated eye. Note the thin capsular wall with loose arranged collagen fibers as compared to the control eye (fig. 12). Arrowheads depict capsular thickness. (Trichrome stain x160)

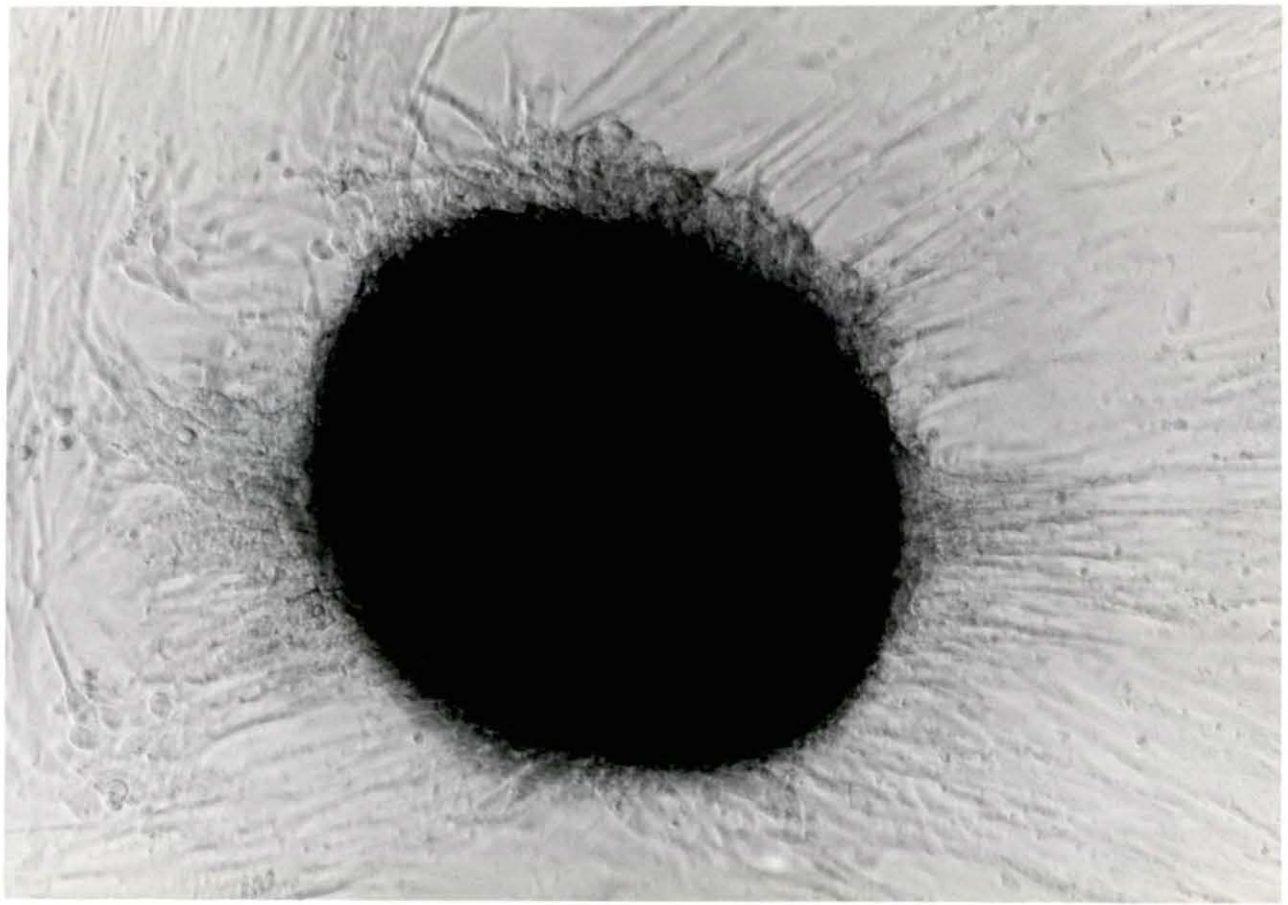

Fig. 14 This phase contrast enhanced photograph depicts a central island of explanted tissue which has adhered along its ventral surface to the tissue culture flask. Surrounding the explant are numerous fibroblast which have undergone replication and subsequent migration outward along the surface of the flask. The photograph was taken 8 days following explantation of the tissue. (x75)

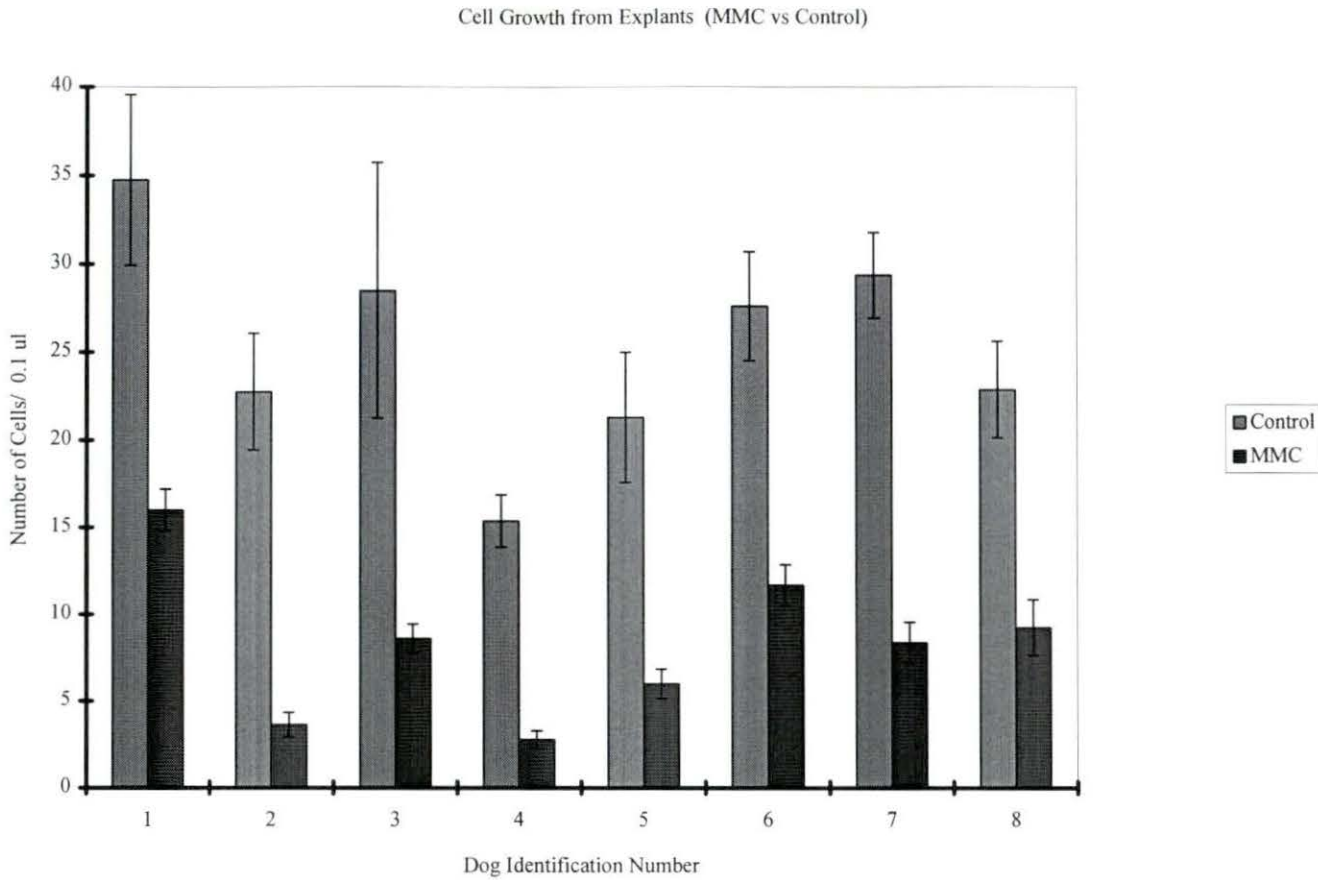

Fig. 15 Histographic comparison of the mean cell counts (cells/ 0.1ml) from control and MMC treated eyes are depicted for dogs 1-8. Cell counts from control eyes were significantly greater than those from MMC treated eyes ($p<0.0001$; Student *t* test). Vertical bars represent the standard error of each mean.

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4. GENERAL CONCLUSION

Results obtained from the *in vitro* trials support a dose dependent inhibitory effect of 5FU and MMC on cultured canine subconjunctival fibroblast proliferation. Additional inhibitory effects on canine subconjunctival fibroblast migration were less clearly established as conflicting results were obtained from F-actin and agarose droplet motility assay studies. While examination of 5FU or MMC treated F-actin filaments revealed structural alterations associated with impaired cytoplasmic adhesion and cellular migration, similar antimetabolite induced inhibitory effects were not obtained when evaluating the role of serum-borne chemoattractants on cellular migration. These findings provided the framework for the subsequent *in vivo* clinical trials.

There are limitations when applying cell culture findings to *in vivo* therapy in eyes undergoing glaucoma filtration surgery. Nevertheless, similar observations were obtained when comparing earlier *in vitro* results to current *in vivo* findings. When compared to control eyes, MMC treated eyes were associated with lower IOP and larger, more pale filtration blebs with a higher incidence of microcyst formation. Mitomycin C treatments also resulted in histologically thinner capsules composed of more loosely arranged collagen fibers. Single-dose MMC therapy was shown to provide continued long-term inhibition of canine cultured subconjunctival fibroblasts. A significantly higher incidence of self-resolving anterior segment complications were associated with intraoperative MMC therapy. While sample size remains small, ongoing clinical trails using glaucomatous canine patients suggest that a reduction in the MMC dose is associated with an absence of all complications encountered in the *in vivo* component of this project. The author encourages continued investigation into this area in an effort to determine the long-term effects in the management of canine glaucoma patients.