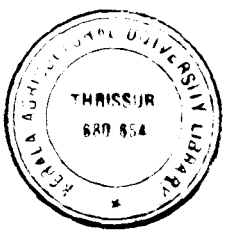
EFFICACY OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGION COUNT TEST FOR *in vivo* BIOMATERIAL EVALUATION

By

P. R. UMASHANKAR



THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
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Centre of Excellence in Pathology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
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KERALA, INDIA

2001

DECLARATION

I hereby declare that the thesis entitled **"EFFICACY OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGION COUNT TEST FOR *in vivo* BIOMATERIAL EVALUATION "** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy
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CERTIFICATE

Certified that this thesis, entitled **"EFFICACY OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGION COUNT TEST FOR *in vivo* BIOMATERIAL EVALUATION"** is a record of research work done independently by **P. R. UMASHANKAR** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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Above all, my humble submission before the Beholder of eternal truth.

Dr. P. R. Umashankar

Dedicated to
my wife and child

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INTRODUCTION

1. INTRODUCTION

Biomedical materials or devices are intended to benefit patients who generally have an anatomical, biochemical or physiological disorder and therefore use of the material or device should not impose any unnecessary adverse effect upon the patient. They belong to three basic classes of materials namely metals, polymers and ceramics which are distinguished according to the nature of their inter atomic bonding. They are pharmacologically inert substances and must be compatible with the body besides being non-toxic. They usually closely duplicate the properties of the tissue they replace. Biomaterials are selected because of their certain characteristics generally described under the collective heading of biocompatibility. Biocompatibility is the term used to describe the state of affairs when a biomaterial exists within a physiological environment, without either the material adversely and significantly affecting the body, or the environment of the body adversely and significantly affecting the material. A biocompatible device or material should perform as intended and present no significant harm to the patient. One important aspect

of biocompatibility is the potential toxicity of the biomaterial. This is due to the migration of an ingredient from the device to the tissue or to a solution that is then administered to the patient. The quantity and type of this ingredient determine the toxicity of the biomaterial. Testing of this toxicity of biomaterials is aptly called safety testing, as a dose response relationship is practically impossible to determine. There are various biological tests both *in vitro* and *in vivo* that evaluate the toxicity or safety of a new biomaterial. These tests are done either on the material directly or on the extracts of the material. *In-vivo* tests based on International organization of standardization (ISO) standard ISO 10993-1:1992 done directly on the materials include systemic acute toxicity test, short term implant test, long term implant test, sensitization test and carcinogenicity test.

Intramuscular implantation in rat gluteus muscle is an established short-term method to evaluate the reaction of tissues at both macroscopic and microscopic levels to a test material that is surgically implanted for seven to 30 days (Cholvin, 1986). Muscle is a highly vascularised tissue and it

possesses vascular transport mechanisms that are more capable of responding vigorously to the presence of reactive foreign material with less toxic results than in less vascular subcutaneous tissue. More over the tissue response in muscle is much more cellular than in subcutaneous tissue. The histopathological parameters studied in short term implantation test around the implant are usually extent of reactive area, concentration of cellular response, types of cellular response, necrosis, oedema, fibrosis and hemorrhage. By evaluating these and giving each response appropriate weightage, a material can be graded for its biocompatibility (Woodward and Salthouse, 1986).

Intramuscular implantation always initially leads to a stage of acute inflammation, which is due to the surgical procedure itself lasting for 24-48 hours. This is followed by a sequence of cellular reactions starting with mobilization and activation of granulocytes, macrophages, lymphocytes and later of fibroblastic cells. Granulocytes are activated within hours while macrophages take a few days to invade the tissue. Macrophages once get activated, can secrete monokines that affect fibrogenesis, angiogenesis,

inflammation and the immune response. Concomitant with the events of acute inflammation, healing response also starts. In the healing response, angiogenesis with infiltration of fibroblasts derived from existing fibrocytes or by differentiation of mesenchymal stem cells occurs into the peri-implant area. After migration there is a period of intense mitotic activity of fibroblasts. These large plump cells become active in the synthesis and secretion of extra cellular matrix components like glycosaminoglycans (GAG) found covalently linked to protein forming proteoglycans, fibronectin and collagen type I and III. Collagen fibers are embedded in the gel matrix provided by GAG and proteoglycans. There is also continuous collagen deposition and concomitant degradation of collagen called remodeling where much of the type III collagen is replaced by type I. Collagenases responsible for this are produced by macrophages and fibroblasts. Collagen content in the wound reaches its maximum by about three weeks time. Following biomaterial implantation the healing process noticed is same as in normal wound healing except for the fact that the sequence of events follows a slower course. The granulation tissue thus developed in the healing process adjacent to implants is

related to the size, surface characteristics as well as their degree of chemical inertness. The capsule thickness and its development time provide an index of material compatibility. This capsule is formed mainly of collagen elaborated by fibroblasts. Recent studies also attributed to fibroblasts a larger and richer role in the complex multicellular response seen in wound healing. Hence logically, the extent and state of fibroblast proliferation and their evaluation should yield information about the amount of collagen synthesized thereby giving an insight into the tissue compatibility of the implanted material.

Recently a number of techniques have been employed in cell proliferation study. They include immuno- histochemical techniques, bromodeoxyuridine incorporation, tritiated thymidine labeling, DNA flow cytometry, mitotic index and Argyrophilic nucleolar organizer region (AgNOR) count technique. Of these AgNOR count is a simple technique and it can be easily adapted to routine biomaterial evaluation.

Nucleolar organizer regions (NOR) are the genomic DNA segments encoding for ribosomal RNA, which can be visualized in chromosome preparations and in interphase nuclei by silver staining. The frequency of

NORs per nucleus may reflect the cell ploidy. AgNOR count is known to correlate with the proliferative activity of the cell populations in normal and malignant tissues.

Fibroblast proliferation state studied using the above histochemical technique along with the presence and concentration of other cellular elements will yield precise information about the biocompatibility of the implant. Fibroblast proliferation state expressed as AgNOR count should be able to detect even subtle differences in degree of biocompatibility in biomaterials that are already found biocompatible on routine techniques. No systematic study has so far been carried out to assess the biocompatibility of implants utilizing AgNOR count technique. The present investigation was therefore taken up with the following objectives:

1. To assess the efficacy of AgNOR count test for Biomaterial evaluation using rat as a model.

2. To compare the AgNOR count test with collagen estimation and histological response in short-term implantation test in the same model.

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

2.1 Biomaterials

Hench and Ethridge (1982) classified biomaterials based on the types of tissue response elicited. They were 1. Inert materials with smooth surfaces 2. Inert materials with microporous surfaces 3. Materials with controlled reactive surfaces and 4. Bioresorbable materials.

Von Recum (1986) added two more classes to the list as Biological and tissue engineered materials.

Williams (1987) defined biomaterials as a non-viable material used in a medical device intended to interact with biological systems possessing a combination of physical, chemical, mechanical and biological properties that rendered it for safe, effective and reliable use within a physiological environment that was both extremely hostile and yet sensitive to and unforgiving of irritating bodies.

Szycher (1992) grouped biomaterials based on the application as materials for reconstructive/plastic surgery for soft tissue/hard tissue application, Cardiovascular applications, specialized applications

like artificial sphincters and implantable controlled drug release devices.

2.2 Biocompatibility

Williams (1981) described biocompatibility as a state of affairs when a biomaterial existed within a physiological environment without either the material adversely and significantly affecting the body or the environment of body adversely and significantly affecting the material.

Lawrence (1986) opined that the toxicological profile of all new materials intended for biomedical application should be evaluated utilizing a battery of tests to determine and delineate its probable scope of safe application. He also described the various reasons for biological incompatibility. According to him the incompatibility was due to the release of biologically active leachable substances from the device, physical contact of the material particularly with regard to thrombosis and cancer induction and biodegradation of materials altering its physical or compatibility properties. The attributes of a biomaterial for being compatible with the body were described by Szycher (1992). He suggested that the material should not cause

uncontrolled thrombosis, damage blood cellular elements, alter blood proteins, destroy or denature enzymes, deplete electrolytes, cause adverse immune response, damage adjacent tissues and cause carcinogenic, mutagenic or teratogenic effects. He also noticed it as the inherent ability of a biomaterial to appropriately interact with host in a specific application.

2.3 Biocompatibility testing.

The battery of tests that were conducted on biomaterial was based on the standards laid down by various international organisations. The first official testing procedure for materials for biomedical application came in United States Pharmacopoeia in 1965. The American Dental Association published "Recommended Standard practices for biological evaluation of dental material in 1972. (John, 1972). The medical Surgical Manufacturers Association published a guideline on medical devices in 1978. (HIMA Report, 1978). Organizations such as American Society of Testing and Materials (ASTM,1984) and International Organization for Standardisation (ISO,1989) subsequently came up with standards for biomaterial testing. As per ISO (1989) the devices were broadly

divide into two broad categories for biocompatibility testing based on the actual site of implantation or use and the characteristics of the tissue in contact with them. The categories included were Implanted materials/devices and externally communicating implanted devices. Implanted materials/devices were again sub grouped into implanted device for use in bone and joints, blood, neural tissue and tissue fluid. Externally communicating implanted devices were categorized into body tissue and fluid devices and blood path direct -single exposure and multiple exposure devices.

The biocompatibility testing based on ISO divided the test methods broadly into *in vitro* and *in vivo* methods. The in vitro methods comprised of 1. Cytotoxicity tests which demonstrated the potential toxicity of extractable and diffusible components of implant material to cloned or differentiated cells in culture. Rae (1986) used both primary and established cells like fibroblasts, tumour cells and embryonic cells for cytotoxicity testing. The cell features evaluated included cell morphology, cell viability/ cell death, cell adhesion to material surfaces and cell growth in the presence of the material (Pizzoferatto *et*

al, 1991). 2. Haemocompatibility tests generally done under this were thrombosis (Hall *et al*, 1989; Ito *et al*, 1990), haemolysis (Singh *et al*, 1990), effect on haemostasis and effect on the complement system (Cenni *et al*, 1991) and effect on formed blood elements (Gilchrist, 1996). 3. Mutagenicity tests: This involved the application of mammalian or non-mammalian cell culture techniques for the determination of gene mutation, changes in chromosome structure and number and genotoxicities (Ames *et al* , 1975; Maron & Ames, 1983; Forster, 1986).

The *in vivo* methods recommended by ISO (1989) included a) Systemic acute toxicity tests intended to determine the biological response of the mouse to a single dose intravenous or intraperitoneal injection of an extract of the sample b) Short term implant test to evaluate the reaction of living tissue at both microscopic and macroscopic levels to a sample that was surgically implanted in an appropriate tissue site in an animal for 7-30 days. It was carried out usually in non-specific soft tissue site such as muscle or subcutaneous space. C). Long term implant test to evaluate the reaction of living tissue at both macroscopic and microscopic levels to a sample that was

surgically implanted in an appropriate tissue site in an animal for periods of six months to two years. D) Sensitisation to estimate the potential for sensitisation either through direct contact or through diffusion of components of implants. E) Neoplasia/ Carcinogenicity test intended to determine the neoplastic/carcinogenic potential of samples either from single or multiple exposures over a period of the total life of the test animals.

Szycher (1992) outlined the reasons for biocompatibility testing. These were 1. to identify any adverse reactions that may lead to failure or which may contribute to un-acceptable clinical outcome, 2. to determine if devices constructed of new materials or process function as intended under simulated use conditions and 3. to advance new technology.

2.4 Intramuscular implantation and histopathology

Intramuscular implantation in rat gluteus muscle was utilized as a short-term method to evaluate the reaction of tissues at both macroscopic and microscopic levels to a sample that was surgically implanted for seven to 30 days. (Cholvin,1986). He reported that muscle being highly vascularised possessed vascular

transport mechanisms that were more capable of responding vigorously to the presence of reactive foreign materials with less toxic results than in less vascular subcutaneous tissue. Moreover the tissue response in muscle was found to be much more cellular than in subcutaneous tissue.

The histopathological parameters studied in Short-term implantation test around the implant were the extent of reactive area, type and concentration of cellular response, necrosis, oedema, fibrosis and haemorrhage. (Woodward and Salthouse, 1986). They observed a stage of acute inflammation lasting for 24-48 hours following intramuscular implantation. This was due to the surgical procedure itself. They also noticed a sequence of cellular reactions starting with mobilization and activation of granulocytes, macrophages, lymphocytes and later by fibroblasts. The granulocytes were seen accumulated within hours after implantation while macrophages took a few days to invade the tissue.

Williams (1989) studied the sequence of events following the application of an implant. He observed adhesion of proteins on to the surface of the material which was followed by acute inflammation and repair.

This reaction and ultimate fate of the implant were found to be influenced by the factor related to the material itself as well as the cells in the surrounding tissue. (Henson,1980; Salthouse,1984; Harbell,1986 Lerd,1986; Anderson,1988; Ziats *et al.*,1998 and Cheroide *et al.* , 1991)

Alison (1992) noted that macrophages once activated, could secrete monokines which affect fibrogenesis, angiogenesis, inflammation and the immune response. He also observed that concomitant with the events of acute inflammation, healing response was initiated. He recorded fibrin products, collagen peptides, fibronectin, platelet derived growth factor, and transforming growth factor β as some of the fibroblastic and angiogenesis chemotactic principles known to operate as mediators. Intense proliferative activity of fibroblast with synthesis and secretion of extra cellular matrix components like glycosaminoglycans (GAG) found covalently linked to protein forming proteoglycans, fibronectin and collagen type I and III was noticed by him.

Collagen content in the wound reached its maximum by about three weeks time (Alison, 1992).

Falck (1995) reported that the cellular response to the material at the tissue-material interface as one of the important factors in determining the biocompatibility of implanted material. Short term intramuscular implantation test as recommended by ISO document 10993-1 (ISO, 1992) permitted assessment of local pathological effects on living tissue at both gross and microscopic level and document 10993-6 provided guidelines for the proper execution of tests and for the correct interpretation of the results(ISO 1992).

2.5 Collagen estimation

Salthouse and Maltaja (1983) reported that the extent of fibroblast proliferation and collagen elaboration was related to the implant size, their degree of chemical inertness and surface characteristics. The capsule around the implant was formed mainly of collagen elaborated by proliferating fibroblasts and hence excess collagen deposited accounted material compatibility (Woodward and Salthouse, 1986). Cholvin (1986) and Ryhanen *et al*(1998) observed that the capsule thickness and its development time provided an index of material compatibility. Reddy and Enwemeka (1996) estimated the

amount of collagen secreted at the repair site by measuring hydroxyproline content.

2.6 Cell proliferation and AgNOR count

Lewin (1980) observed the presence of NORs on the acrocentric chromosomes 13,14,15, 21 and 22 of humans. Albert *et al.* (1983) reported nucleolar organizer regions (NORs) as genomic DNA segments encoding for ribosomal RNA. Ploton *et al.* (1986) developed a single step silver staining method for demonstration of AgNOR and their counting in which they observed organizer regions as dark circumscribed dots in the nucleus. Stoschek and King (1986) reported changes in distribution of NORs to mirror disorganization of nucleolus and increased ribosomal transcriptional activity and cellular protein synthesis. AgNORs could be visualized in chromosomes preparations and in interphase nuclei by silver staining as each NOR was associated with Argyrophilic proteins such as RNA polymerase 1, C-23 (Nuclolin) and B=23 (Nematrin) (Crocker and Nar, 1987). Dervan *et al.* (1989) observed variations in the AgNOR counts of benign lesions and carcinomas. In benign lesions the mean AgNOR count per cell was 2.65 to 6.8 and in carcinomas it ranged from 4.6 to 26.9. AgNOR technique was found to be simple and

successfully applied on formalin fixed paraffin embedded tissue for cell proliferation studies by various authors. (Bratulic *et al.*, 1996, Prakash *et al.* 1997; Korkolopoulou *et al.*, 1998). Prakash *et al* (1997) and Korkolopoulou *et al.* (1998) observed a good correlation between the AgNOR count and other advanced techniques like PCNA or Ki 67 immuno-histochemistry.

Chappard *et al.* (1998) and Kashyap *et al.* (1998) opined that AgNOR count could be utilized for cell proliferation studies as it correlated well with the proliferative activity of the cell populations, whether normal or malignant. According to Barzilai *et al.* (1998) NOR staining represented actively transcribing NORs and thus rDNA. After silver staining they could be observed as back dots (AgNORs) in the nuclei. They demonstrated that the size and number of AgNORs reflected the cell and nuclear activity and the state of proliferation. Lorand -Metze *et al.* (1998) observed that the number of clusters and the dots in the silver stained nuclei were related to the percentage of cells in synthetic phase. They also observed the AgNOR area to be related to the cell replication time and mean size of AgNORs were found to be smaller in highly proliferating cells. Underwood, (1992) and

Montaner *et al.* (1998) observed that the NORs could be demonstrated by *in situ* hybridisation using radiolabelled rRNA and by immuno-histochemistry using antibodies against NOR proteins.

Dover (1992), Bratulic *et al.* (1996), Hussain *et al.* (1997), Kiupel *et al.* (1998), Krishnamurthi and Paliwal (1998), Oyama *et al.* (1998) Sharma *et al.* (1998), Sinha *et al.* (1998) and Piffko *et al.* (1999) used single step silver staining procedure for AgNOR for studying different neoplastic conditions in humans and animals. Various other tests utilised for studying the cell proliferation were immuno-histochemisty of Ki 67 antigen (Ki 67 labelling index), Proliferating cell nuclear antigen (PCNA), expression protein of p53 gene, HMB-45, NKI-c3, anti-bcl-2onoprotein, p125 antigen, DNA polymerase δ and α, K112 antigen, bromodeoxyuridine incorporation, tritiated thymidine labelling, DNA flow cytometry and mitotic index. (Grigolato *et al.* ,1997; Prakash *et al.* 1997; Korkolopoulou *et al.*, 1998; Kiupel *et al.*, 1998; Oyama *et al.* 1998; Sharma *et al.* 1998; Sinha *et al.* 1998; Piffko *et al.* 1999).

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Biomaterial

1. Reactive biomaterial: Medium chromic catgut absorbable suture (2/0) was used as reactive biomaterial.

2. Non-reactive biomaterial: Poly propylene non-absorbable suture (2/0) was used as non-reactive biomaterial.

3.2 Experimental animals

Forty eight adult Wistar rats of either sex, weighing 200-250g were used for the implantations. The animals were given standard rat feed and clean water *ad libitum* during the study. Stipulations laid down under Prevention of Cruelty to Animals Act (Amendment 1998) were followed. Care and management of the animals were done according to the guideline for care and use of animals in scientific research (2000) issued by Indian National Science Academy, New Delhi.

3.3 Experimental design

The animals were randomly divided into two major groups, Group I of 24 animals for reactive biomaterial

and Group II of 24 animals for non reactive biomaterial. Each Major group consisted of two study durations (seven days and 14 days). Each major group contained two test subgroups and two control subgroups. The test and control subgroups groups were 1. Histopathology subgroup and 2. Collagen estimation subgroup. (Table 1.)

Table 1 Experimental design

Study duration (Days)	Reactive biomaterial (Number of animals)				Non reactive biomaterial (Number of animals)			
	Histopathology		Collagen estimation		Histopathology		Collagen estimation	
	Test	Control	Test	Control	Test	Control	Test	Control
7	3	3	3	3	3	3	3	3
14	3	3	3	3	3	3	3	3

In the test animals the biomaterial was implanted bilaterally into the gluteus muscle aseptically. In the control animals bilateral sham surgery was performed. At the end of the study, the animals were

sacrificed and tissue samples were collected with each animal providing two samples.

3.4 Procedure

3.4.1 Implantation of biomaterial

The aseptic surgery was done under general anaesthesia. Anaesthesia was induced by intramuscular injection of Ketamine hydrochloride[a] (50mg/Kg) and Xylazine hydrochloride[b] (5mg/Kg) combination and animal were controlled on ventral recumbence. The surgical site was prepared by shaving and scrubbing with Betadine surgical scrub[c]. Implantation was done after making a skin incision and the biomaterial in suture form was threaded on a half circle 25mm round bodied needle. A bite was taken on the gluteus muscle after exposing the muscle. The suture ends on either side was cut off retaining the biomaterial in the gluteus

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a- Ketmin 50: Ketamine hydrochloride 50 mg /ml, Themis Chemicals, Mumbai.

b- Xylaxin: Xylazine hydrochloride 20 mg / ml, Indian Immunologicals, Hyderabad.

c- Betadine surgical scurb: Povidone iodine 7.5 per cent w/v, Win-Medicare, New Delhi

muscle. The skin incision was closed by simple apposition using 2/0 silk sutures. The animals were given Oxytetracyline hydrochloride[a] at 25mg/Kg body weight in drinking water for the next five days.

3.4.2 Explantation of Biomaterial

At the end of the study the animals were euthanised using an excess dose of anaesthetic Thiopentone sodium[b] given intra-peritoneally. Autopsies of the animals were done. Biomaterial along with the surrounding tissue was collected and immediately stored at -80°C for collagen subgroup samples and histopathology subgroup samples were fixed in 10 per cent neutral buffered formalin. In the sham group, the muscle alone at the surgery site was explanted and fixed in 10 percent buffered formalin.

3.4.3 Estimation of collagen

Tissue collagen of the peri-implant area was estimated using the method described by Reddy and Enwemeka (1996).

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a- Terramycin 250: Oxytetracyclin 250 mg capsule, Pfizer, Mumbai

b- Pentothal: Thiopenton sodium 1 g, Abbot Laboratories, Mumbai

3.4.3.1 Collection and preparation of sample

Approximately two gram of the tissue sample along with the implant was collected, washed in saline and was immediately transferred to -80°C and kept for 24 hours. This was followed by lyophilizing the tissue sample for 48 hours. After lyophilisation the tissue samples were stored at -80°C until it was used for collagen estimation. For estimation approximately 100 mg of tissue sample was taken and homogenized in distilled water to give a final tissue sample concentration of 5 mg/mL-distilled water.

3.4.3.2 Collagen estimation

3.4.3.2.1. Chemicals

Chloramine -T, p-dimethylaminobenzaldehyde and L-hydroxyproline were purchased from Sigma Chemical Company. Sodium acetate, citric acid, perchloric acid n-propanol, sodium hydroxide and acetic acid were purchased from Fisher Scientific. All the chemicals were of analytical grade.

3.4.3.2.2 Preparation of reagents

Hydroxyproline standard: A solution containing 2 mg/mL of hydroxyproline was prepared in distilled water.

Acetate-citrate buffer pH 6.5: The buffer was prepared by dissolving 120g of sodium hydroxide in distilled water; pH was adjusted to 6.5 and brought to one liter.

Chloramine T reagent (0.056M): 1.27 g of chloramine T was dissolved in 20 mL 50% n-propanol and brought to 100 mL with acetate-citrate buffer.

Ehrlich's reagent (1M): 15g of p-dimethylaminobenzaldehyde was dissolved in n-proponol/perchloric acid (2:1 v/v) and brought to 100 mL. This was freshly prepared before estimation.

3.4.3.2.3 Assay procedure

Aliquots of standard hydroxyproline (20µg) and test samples were mixed gently with sodium hydroxide (2N final concentration) in a total volume of 50 µL. The samples were hydrolyzed by autoclaving at 120^{0}C for 20 min. 450µL of chloramine-T was added to the

hydrolyzate, mixed gently, and the oxidation was allowed to proceed for 25 min at room temperature 500µL of Ehrlich's aldehyde reagent was added to each sample, mixed gently, and the chromophore was developed by incubating the samples at 65°C for 20 min. Absorbance of each sample was read at 550 nm using spectrophotometer.

3.4.4 Histopathological examination

3.4.4.1 Collection, fixation, processing and sectioning of tissue samples

Gluteus muscle along with implant was excised out and after washing in saline; it was fixed in neutral buffered formalin for one week. The tissue samples were processed and sections of 5µ thickness were cut and stained by routine hematoxylin and eosin as per the method of Sheehan and Hrapchak, (1980)[1] Representative sections from each sample were also subjected to special staining such as Van Gieson's picric acid-fuchsin stain for collagen (Sheehan and Hrapchak,1980), modified trichrome stain for muscle collagen (Bancroft and Stevens, 1977) and AgNOR as per the method of Ploton *et al.* (1986).

3.4.4.2 Hematoxylin and eosin staining and evaluation

The sections were stained with H&E (Sheehan and Hrapchak, 1980) and evaluated for peri-implant necrosis, degeneration, infiltration by neutrophils, lymphocytes, eosinophils, plasma cells, macrophages, fibrosis, presence of giant cells, foreign body debris, fatty infiltration, thickness of reaction zone and scar thickness (Woodward and Salthouse, 1986). The observations were recorded in the format given in Appendix 1.

3.4.4.3 Van Gieson's picric acid-fuchsin stain for collagen and evaluation

The sections were stained with Van Gieson's technique (Sheehan and Hrapchak, 1980) for demonstration of fibrosis, collagen deposition and measurement of scar thickness by micrometry.

3.4.4.4 Modified trichrome stain for muscle collagen The sections were stained by modified trichrome method ((Bancroft and Stevens, 1977) for demonstration of fibrosis, collagen deposition and measurement of scar thickness by micrometry.

3.4.4.4 AgNOR staining

3.4.4.4.1 Stain

a) Silver nitrate solution: 50 per cent solution of AR grade silver nitrate was prepared in de-ionized distilled water and stored in a polypropylene container away from the light.

b) Gelatin solution: 2 per cent Gelatin solution was prepared in de-ionized distilled water and pure formic acid was added to this to a final concentration of 1 percent. De-ionized distilled water was used for the preparation of solution and the prepared solution was stored in a polypropylene container

3.4.4.4.2 Staining

After deparaffinising, the sections were gradually hydrated through graded alcohol and washed in de-ionized distilled water. Slides containing the sections were then placed on the staining rack and two drops of gelatin solution was dropped on the section. Over this one drop of silver nitrate solution was placed and the sections were incubated at room temperature for 45 minutes in darkness. The sections were evaluated for AgNOR count of 100 fibroblast

nuclei using image analysis soft ware Optimas 6 under oil immersion objective. The spindle shaped nucleus and abundant extra-cellular matrix deposition as observed by dark granular deposits identified the fibroblasts. The AgNOR count was expressed for the average of 100 nuclei.

3.4.5. Statistical evaluation

Quantitative data like AgNOR count, scar thickness and collagen content were evaluated for statistically significant difference using ANOVA. Following this unpaired 't' test for equal and unequal variance based on F test result was done. Correlation coefficient between means of AgNOR count and scar thickness was found out and articulated as graph. The mean of data was represented as graphs with standard error (Snedecor and Cochran, 1967).

RESULTS

4 RESULTS

4.1 Implantation

Following implantation all the animals tolerated the implant. There were no symptoms of wound infection or implant-associated infection in any animals at the time of autopsy. All animals completed their respective duration of implantation and were euthanised and autopsy was conducted as scheduled at the end of the study

4.2 Collagen estimation

The collagen content of tissue around the implanted samples of the control and test groups expressed in gram per 100gram dry tissue (lyophilized tissue) is given in Table 2.

The collagen content in the control sample at seven days was 23.8±2.57g/100g-lyophilized tissue and at 14 days it was 20.91± 1.26g/100g-lyophilized tissue. Tissue sample around reactive biomaterial at seven and 14 days were 26.18±0.27 and 22.54 ± 0.29g /100g- lyophilized tissue respectively. Collagen content of the tissue samples around non-reactive

biomaterial was 19.6 ± 4.85 and 23.64 ± 0.37g/100g-lyophilized tissue at seven days and 14 days respectively. The apparent difference found in the collagen levels in different groups (Fig.1) was found not significant statistically.

Table 2. Collagen content in muscle around implanted materials of the control and test groups (g/100g lyophilized tissue)

Days after implantation	Collagen content (g/100g) Mean ± SE		
	Control	Reactive biomaterial	Non-reactive biomaterial
7	23.8±2.57	26.18±0.27	19.6±4.85
14	20.91±1.26	22.54±0.29	23.64±0.37

4.3 Histopathological evaluation

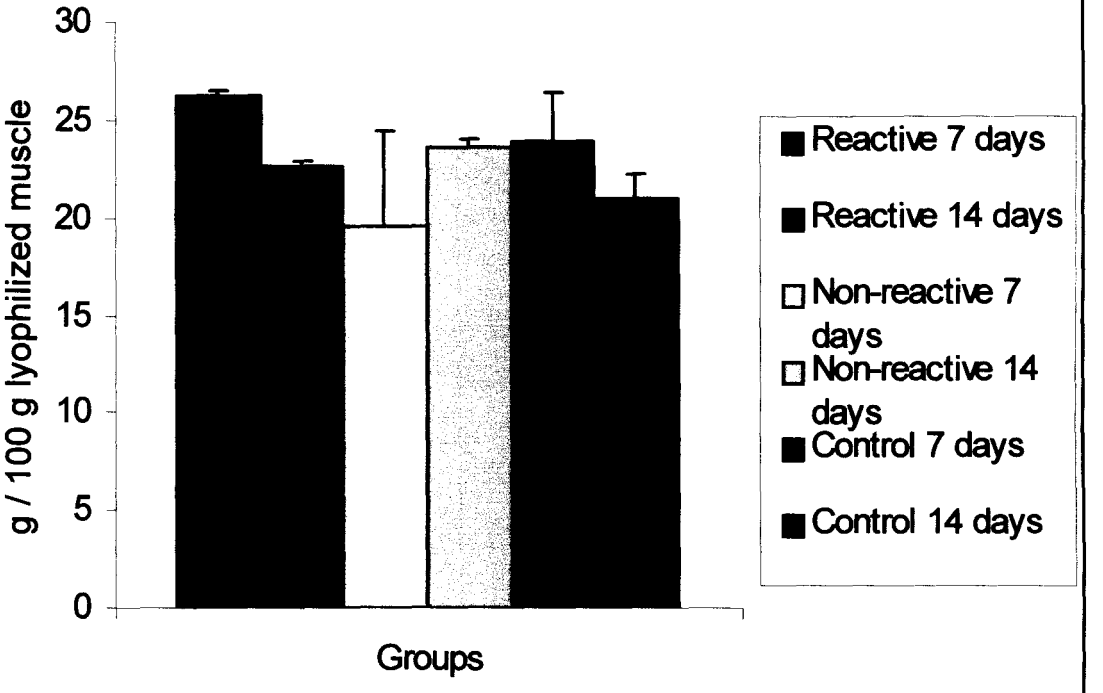
4.3.1 Hematoxylin and eosin staining

The histopathological parameters studied included muscle necrosis, degeneration, hemorrhage, foreign body debris of implant origin, fatty infiltration and neovascularisation in the peri-implant area. The second feature studied was the intensity of cellular infiltrations like neutrophils, lymphocytes,

eosinophils, plasma cells, macrophages, and giant cells. Finally the extent of fibrosis was assessed. The tissue response in individual animals in the same group differed slightly in their cellular responses, but were within a certain limit enabling adequate interpretation. The reaction zone thickness of the same section varied at different regions around the implant. For the ease of interpretation, average thickness recorded of more than six regions around the implant at high power objective fields was used for analysis. Sham sites in the control groups could not be identified at either seven or 14 days.

The sections of tissue around reactive biomaterial (medium chromic catgut) of 7 days post implantation showed a picture of acute inflammation with a thin zone of reaction characterized by moderate neutrophilic infiltration, moderate to severe macrophage infiltration and mild fibrosis (Fig. 2). Occasional lymphocytes and hemosiderin-laden macrophages could be seen. The peri-implant area showed occasional muscle necrosis, neovascularisation and mild hemorrhage. There was no evidence of muscle degeneration, foreign body debris of implant origin or fatty infiltration in the peri-implant area. Plasma

Fig. 1 Collagen content of reactive and non-reactive material implanted muscle in g/100 g lyophilized tissue (Mean of six samples with SE)



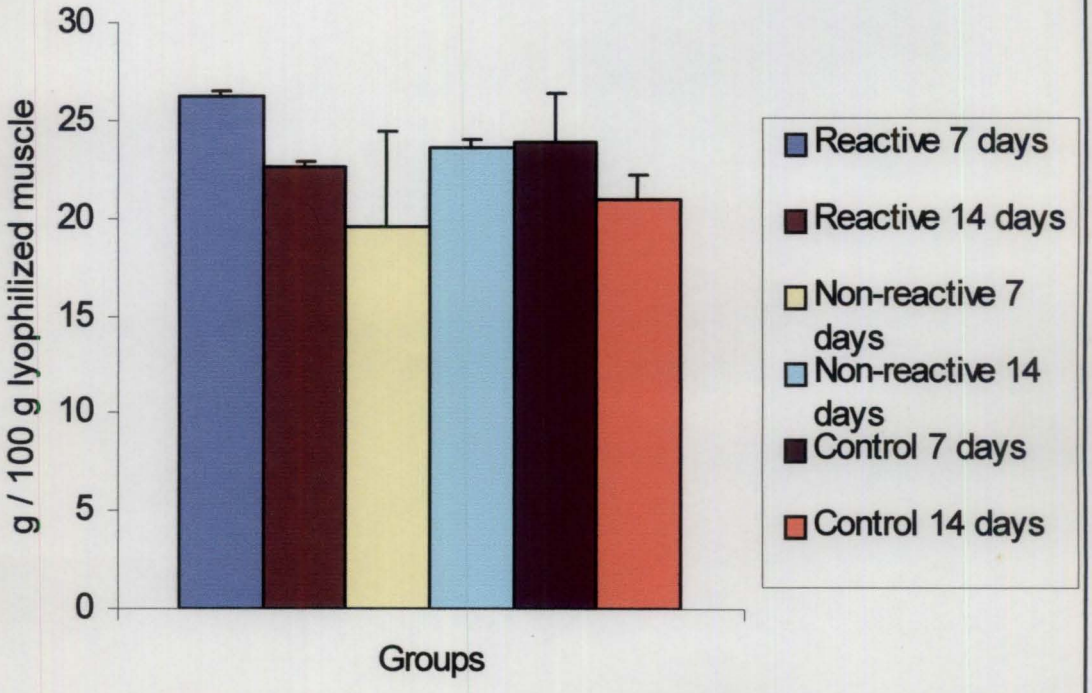
cells and giant cells were absent. Tissue around reactive biomaterial at 14 days post implantation showed a picture of transformation of inflammation from acute to chronic nature with a thick zone of reaction (Fig. 3). The presence of moderate macrophage infiltration, mild to moderate neutrophilic infiltration, mild lymphocyte infiltration and also presence of mild to moderate hemosiderin loaded macrophages characterized this zone. Neovascularisation, occasional muscle necrosis, mild hemorrhage, and fibroblasts in many layers were noted in the peri-implant area. There was no evidence of foreign body debris of implant origin or fatty infiltration in the peri-implant area. Plasma cells and giant cells could not be observed.

Tissue around non-reactive biomaterial (poly propylene) at seven days post implantation showed a picture of acute inflammation (Fig. 4) with a reaction zone a little thicker than that seen around catgut for the same duration. Moderate neutrophilic and macrophage infiltration characterized the reaction zone. Presence of occasional lymphocytes and areas of muscle necrosis with macrophage infiltrating into the necrosed area were observed. Peri-implant area showed

Fig. 2 Reactive material at seven days post implantation: Acute inflammation showing moderate infiltration with neutrophils and macrophages and mild fibrosis **(H&E x 400)**

Fig. 3 Reactive material at 14 days post implantation: Mild chronic inflammation with thick zone of reaction showing mild mononuclear infiltration and moderate to severe fibrosis **(H&E x 400)**

Fig. 1 Collagen content of reactive and non-reactive material implanted muscle in g/100 g lyophilized tissue (Mean of six samples with SE)



cells and giant cells were absent. Tissue around reactive biomaterial at 14 days post implantation showed a picture of transformation of inflammation from acute to chronic nature with a thick zone of reaction (Fig. 3). The presence of moderate macrophage infiltration, mild to moderate neutrophilic infiltration, mild lymphocyte infiltration and also presence of mild to moderate hemosiderin loaded macrophages characterized this zone. Neovascularisation, occasional muscle necrosis, mild hemorrhage, and fibroblasts in many layers were noted in the peri-implant area. There was no evidence of foreign body debris of implant origin or fatty infiltration in the peri-implant area. Plasma cells and giant cells could not be observed.

Tissue around non-reactive biomaterial (poly propylene) at seven days post implantation showed a picture of acute inflammation (Fig. 4) with a reaction zone a little thicker than that seen around catgut for the same duration. Moderate neutrophilic and macrophage infiltration characterized the reaction zone. Presence of occasional lymphocytes and areas of muscle necrosis with macrophage infiltrating into the necrosed area were observed. Peri-implant area showed

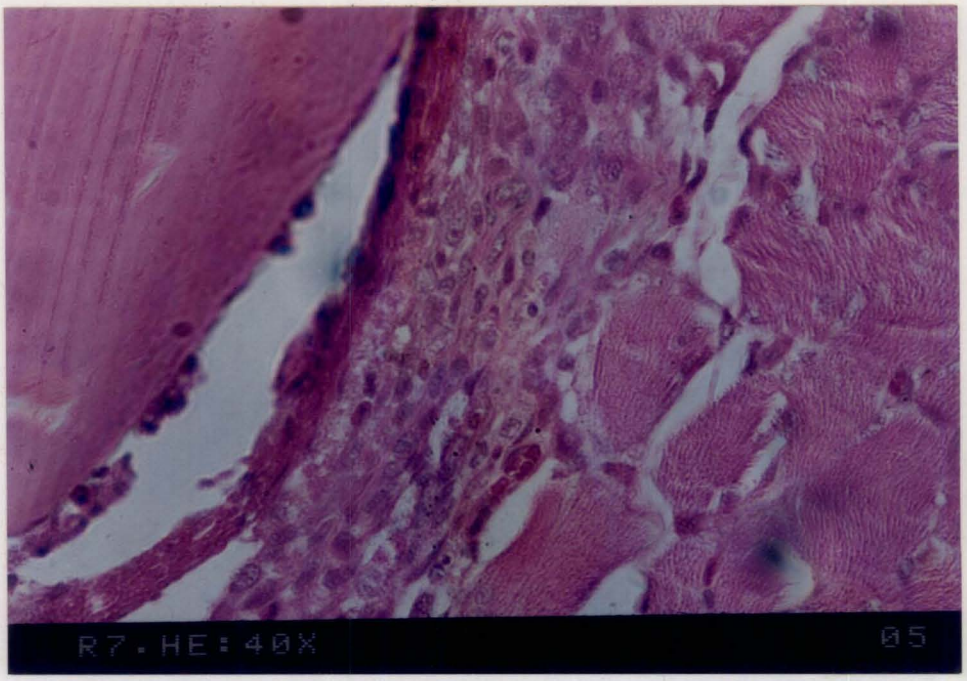
occasional hemorrhages and mild fibrosis. There was no evidence of muscle degeneration, foreign body debris of implant origin or fatty infiltration in the peri-implant area. Fourteen days following implantation a picture of mild chronic inflammation persisted around the implant. The reaction zone had reduced and was much lesser than that seen around the catgut of the same duration. The reaction zone was characterized by the presence of mild to moderate infiltration of lymphocytes (Fig. 5), moderate infiltration of macrophages, mild fibrosis and presence of occasional neutrophils. Peri-implant area showed occasional muscle necrosis with macrophage infiltration in the necrosed area. There was no evidence of foreign body debris of implant origin or fatty infiltration in the peri-implant area. Plasma cells and giant cells were absent at both time periods.

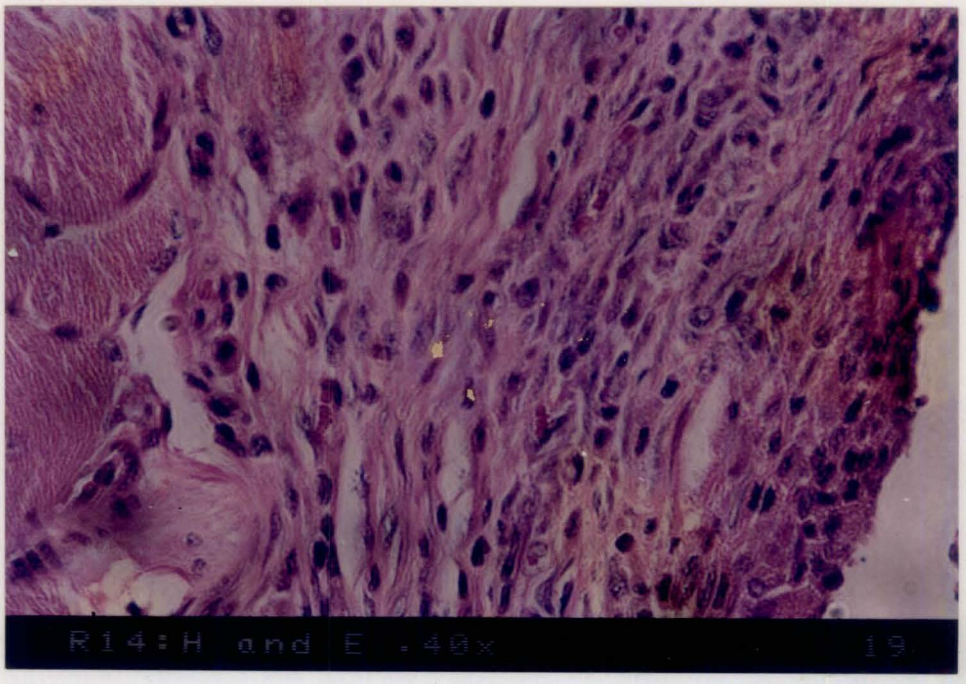
4.3.2 Special stains for collagen: Van Gieson's picric acid-fuchsin stain

The scar thickness could not be measured in this staining, as the scar could not be demarcated from reaction zone. Other parameters observed were fibrosis and collagen deposition. The sections on staining showed intense red coloration of perimysium and also

Fig. 2 Reactive material at seven days post implantation: Acute inflammation showing moderate infiltration with neutrophils and macrophages and mild fibrosis **(H&E x 400)**

Fig. 3 Reactive material at 14 days post implantation: Mild chronic inflammation with thick zone of reaction showing mild mononuclear infiltration and moderate to severe fibrosis **(H&E x 400)**


R7.HE:40X
05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19

the catgut implant wherever present. The peri-implant area of fibrosis and collagen deposition showed less intensity of color compared to the above. Muscle tissue was stained yellow. Sham sites at seven days and 14 days could not be identified in both the controls.

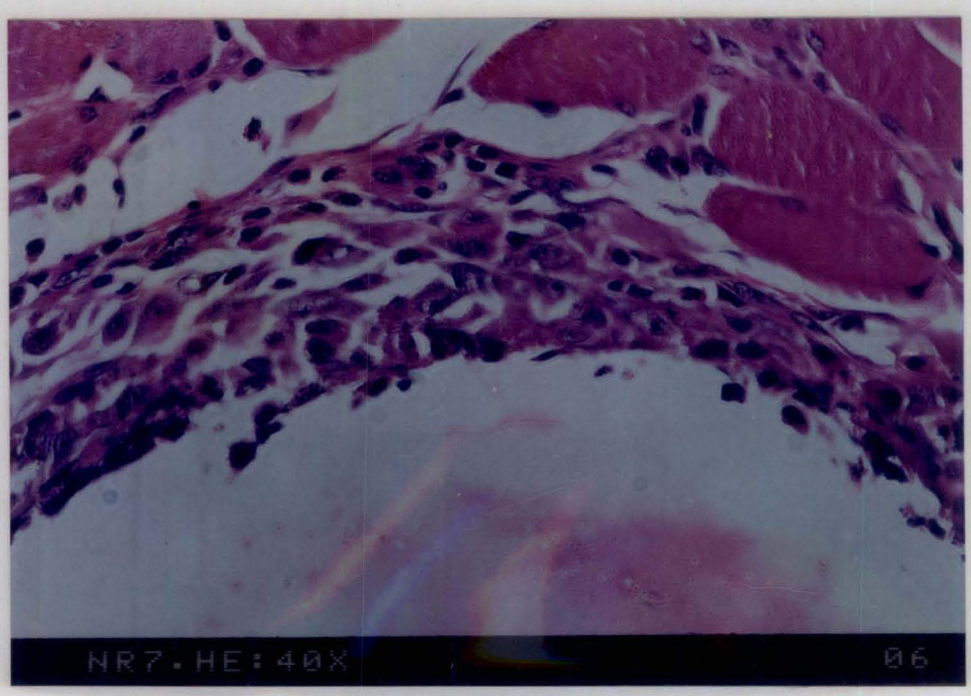
Tissue around reactive biomaterial (medium chromic catgut) at seven days post implantation showed mild fibrosis and mild collagen deposition. (Fig. 6). At 14 days severe fibrosis with good collagen deposition was present (Fig. 7). Sections of non-reactive material (Polypropylene) with surrounding tissue after seven days showed moderate fibrosis and mild collagen deposition (Fig. 8). At 14 days following implantation mild fibrosis with mild collagen deposition was observed (Fig. 9).

4.3.3 Special stains for collagen: Modified trichrome stain for muscle collagen

The extent of fibrosis, collagen deposition around the implant and scar thickness were studied. The sections on staining showed green coloration of the peri-implant area with collagen deposition, areas of fibrosis, perimysium and also the catgut implant

Fig. 4 Non-reactive biomaterial at seven days post implantation: Mild to moderate mononuclear cell accumulation and muscle necrosis **(H&E x 400).**

Fig. 5 Non-reactive biomaterial at 14 days post implantation: Reduced reaction zone showing mild infiltration of lymphocytes and macrophages with fibrosis and peri-implant muscle necrosis **(H&Ex 400)**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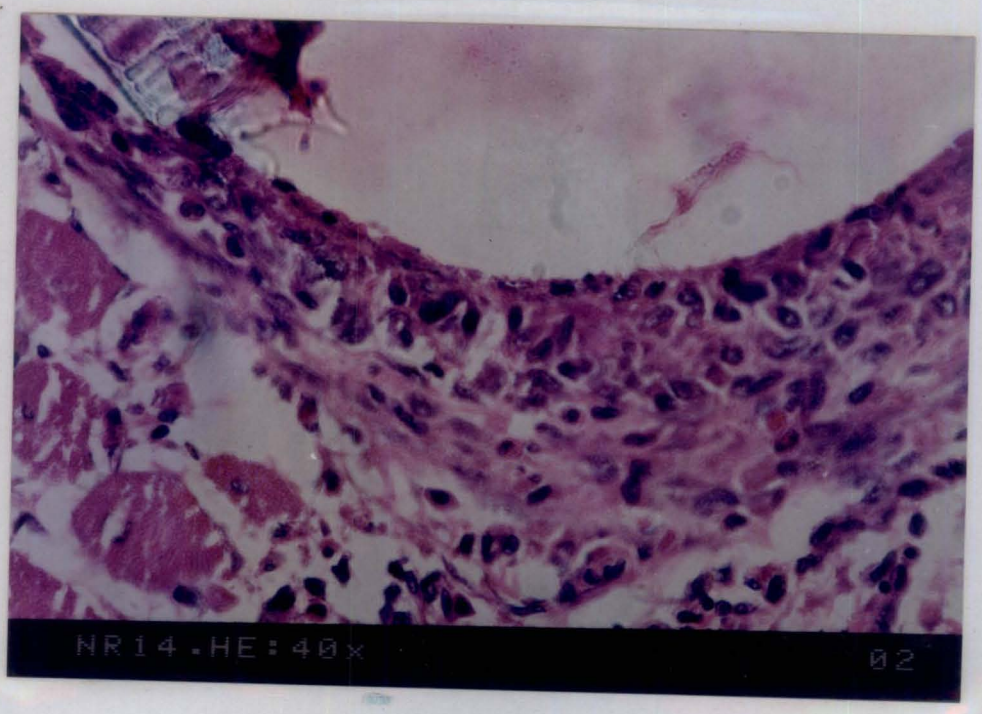
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02

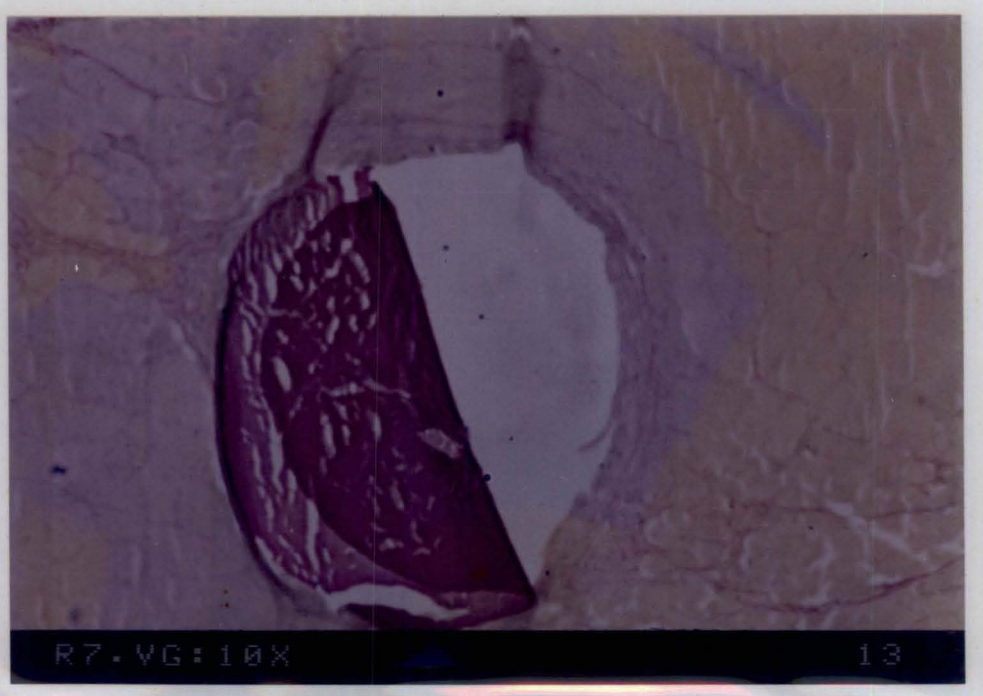
wherever present. The peri-implant muscle tissue appeared dark blue to red in color. The scar thickness could be measured with reasonable accuracy in this staining as the scar was well demarcated from reaction zone. Scar thickness measured (Table 3 and Fig. 10) in each group was subjected to statistical evaluation and correlation coefficient was worked out with AgNOR count (Fig. 11). Both the control sham sites at seven days and 14 days could not be identified.

The tissue around reactive biomaterial (medium chromic catgut) at seven days post-implantation showed mild fibrosis, mild collagen deposition and a mean scar thickness of 56.31 ± 6.58μm (Fig. 12). Severe fibrosis with good collagen deposition was present (Fig. 13) at 14 days post-implantation. The scar thickness noticed was 83.08 ± 6.39μm.

Sections of tissue around non-reactive (Polypropylene) material at seven days post implantation revealed mild fibrosis, mild collagen deposition and a mean scar thickness of 46.9 ± 7.08μm. (Fig. 14). Mild to moderate fibrosis and collagen deposition was noted at 14 days post implantation. The scar thickness noticed was 35.45 ± 5.37μm.

Fig. 6 Reactive biomaterial at seven days post implantation: Fibrosis and mild collagen deposits **(Van Gieson's stain x 100)**

Fig. 7 Reactive biomaterial at 14 days post implantation: Fibrosis and moderate to severe collagen deposition along with mononuclear cell infiltration **(Van Gieson's stain x 100)**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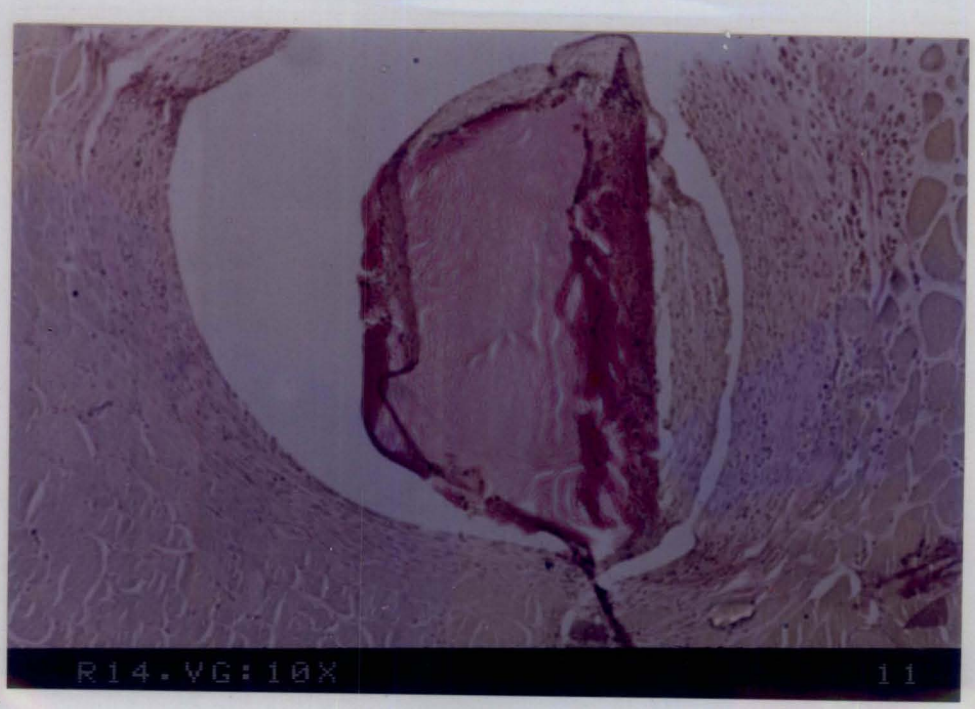
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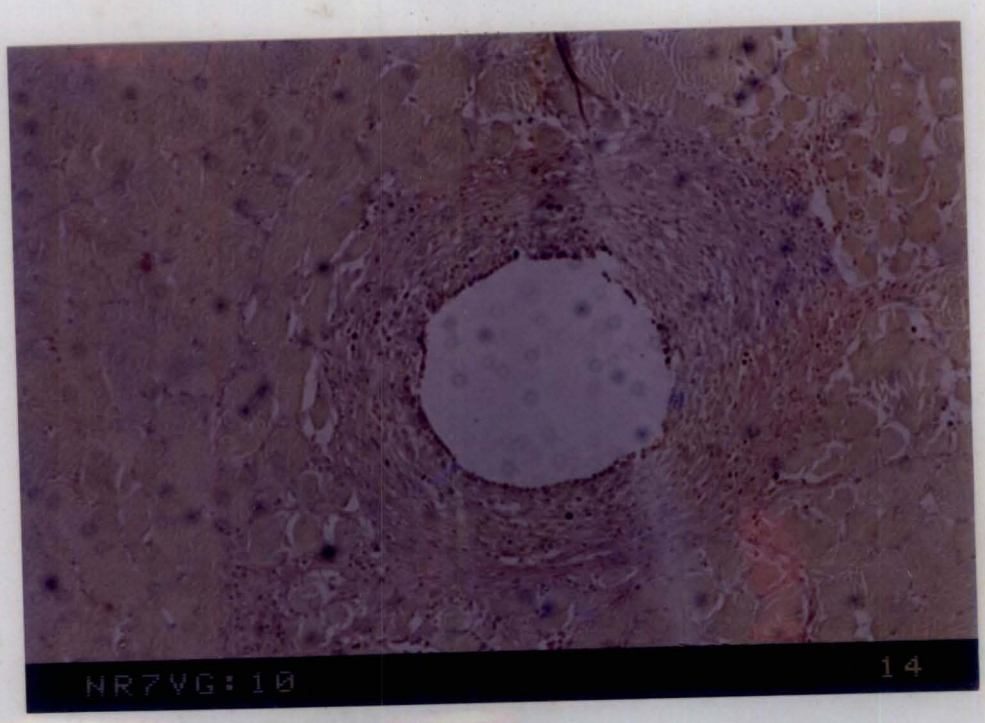
11

Fig. 8 Non-reactive biomaterial at seven days post implantation: Moderate fibrosis and mild collagen deposition **(Van Gieson's stain x 100)**

Fig. 9 Non-reactive biomaterial at 14 days post implantation: Mild fibrosis and mild collagen deposition **(Van Gieson's stain x 100)**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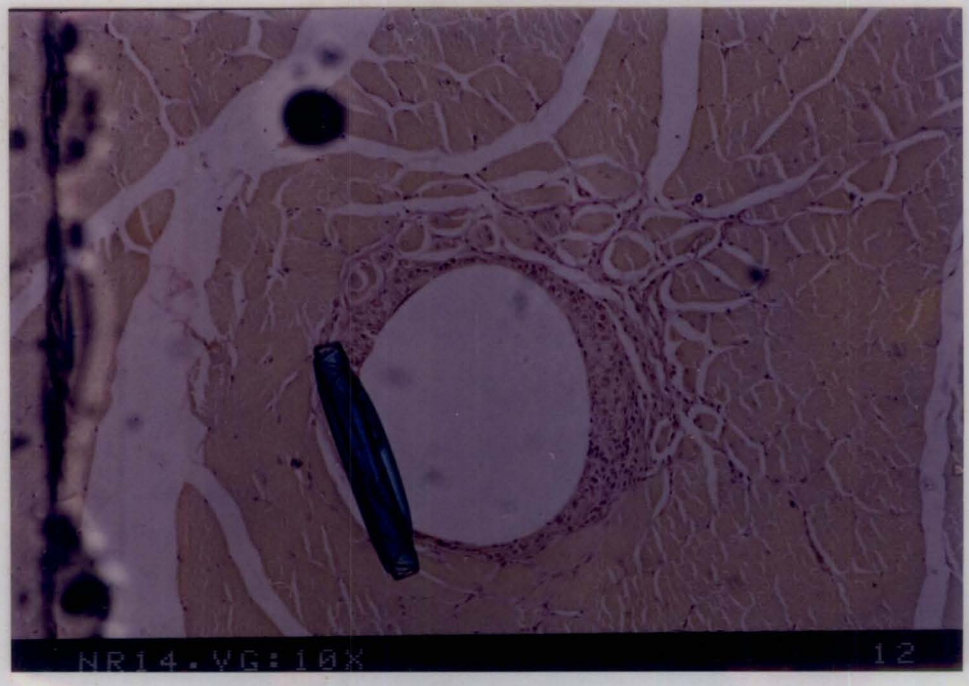
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12

Table 3. Peri-implant scar thickness around reactive and non-reactive biomaterial at seven and 14 days post implantation (n= 6)

Treatment groups Scar-thickness (μm)	Reactive biomaterial (Chromic catgut)		Non-reactive biomaterial (Polypropylene)	
	7 days	14 days	7 days	14 days
Mean	56.31	83.08	46.9	35.45
SE	6.58	6.39	7.08	5.37

Analysis of data on scar thickness showed, statistically significant differences between groups. There was no statistically significant differences between non-reactive seven-day samples and non-reactive 14-day sample. Reactive seven days and reactive 14 days showed statistically significant difference in their scar thickness. Correlation coefficient between means of scar-thickness and AgNOR came to 0.9 at their respective study durations.

4.3.4 AgNOR staining

State of fibroblast proliferation was studied using this staining procedure. Fibroblasts were identified by morphology (cells with elliptical nuclei). Fibroblasts showed abundant extra-cellular

Fig. 10 Peri-implant scar thickness around reactive and non-reactive biomaterial at seven and 14 days post implantation (mean of six samples with SE)

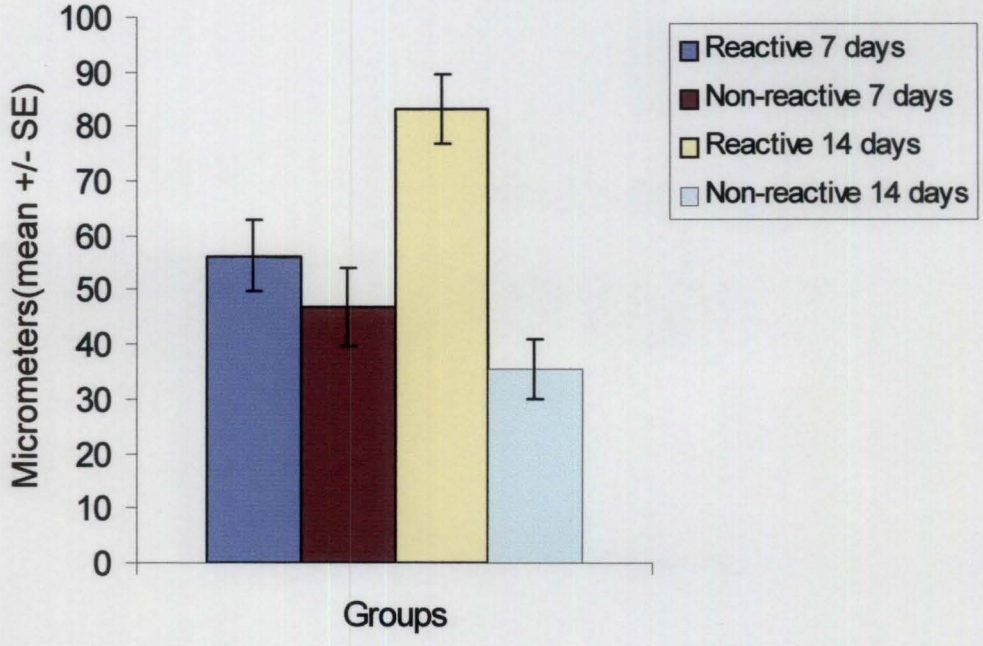


Fig.11 Means of scarthickness and AgNOR count of fibroblast cells around reactive and non-reactive implants at 7 and 14 days post implantation

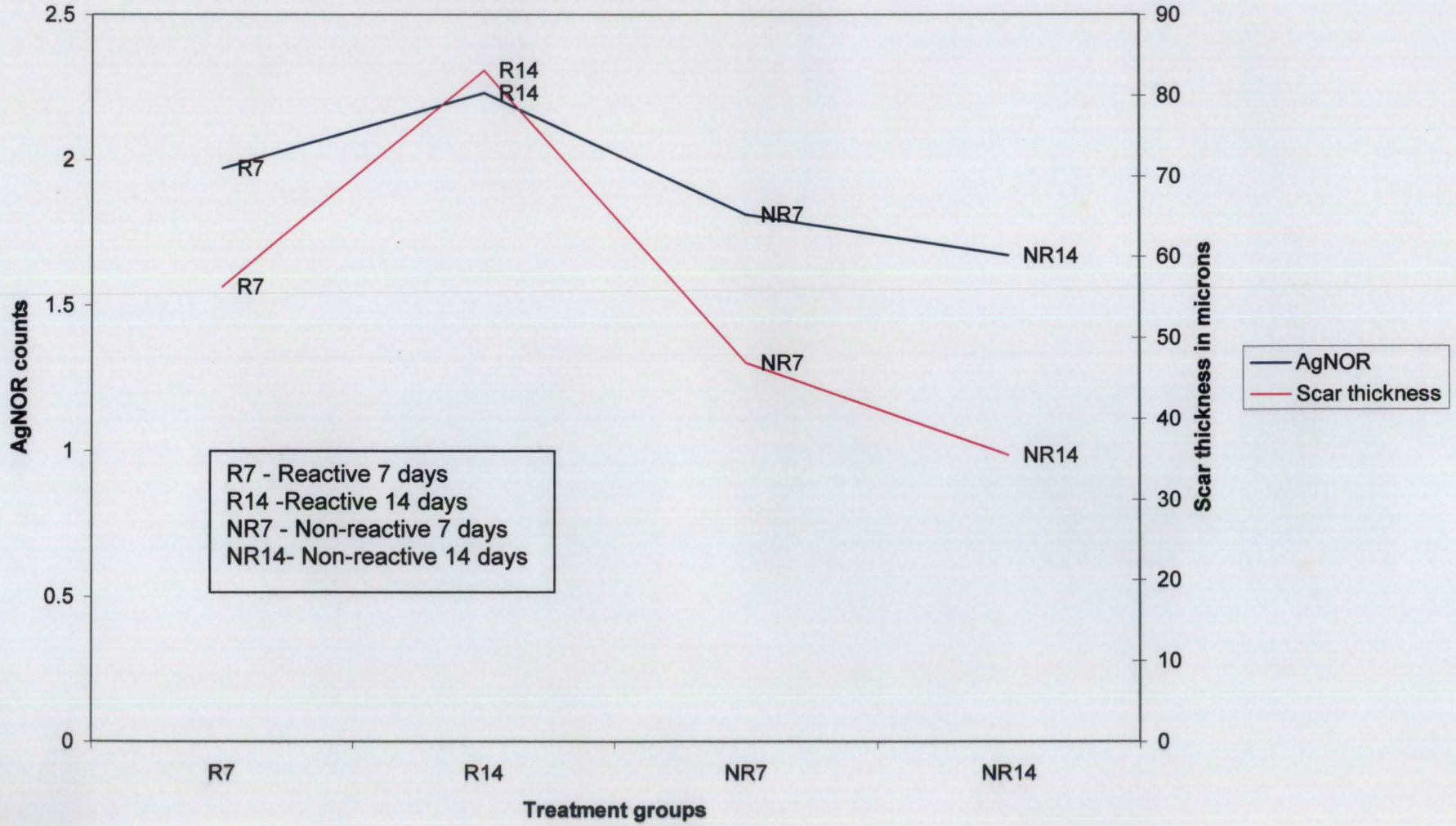
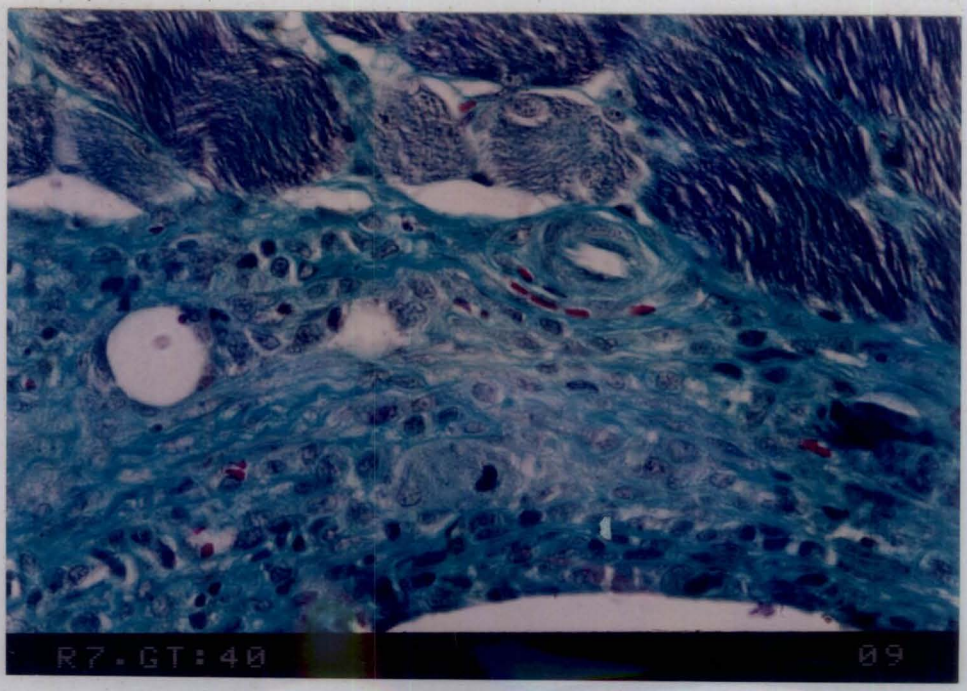
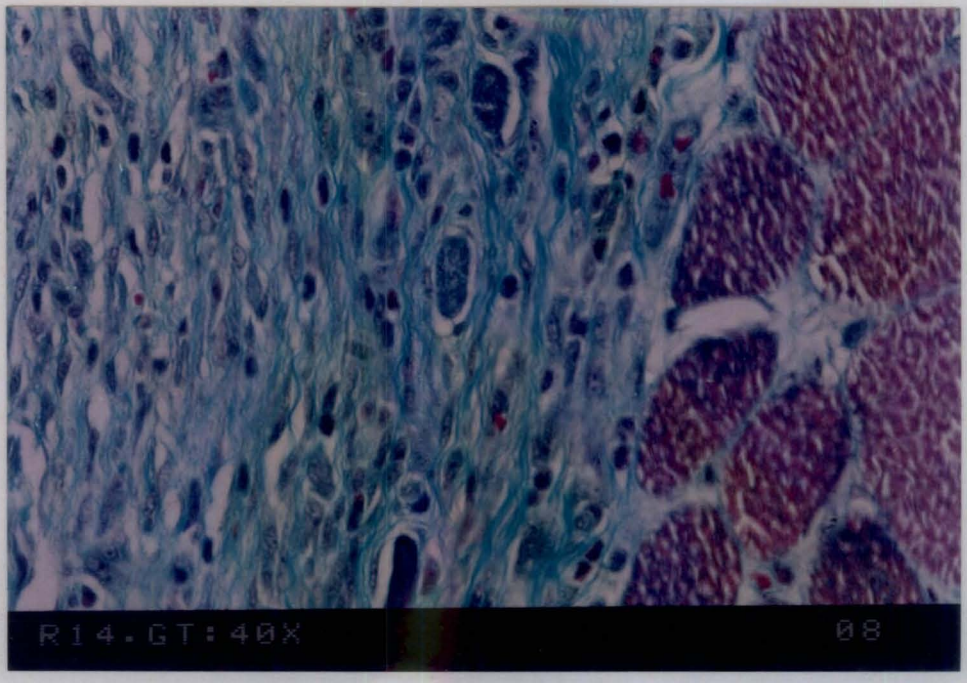


Fig. 12 Reactive biomaterial at seven days post implantation: Mild fibrosis and mild collagen deposition **(Modified trichrome stain x 400)**

Fig. 13 Reactive biomaterial at 14 days post implantation: Severe fibrosis and more collagen deposition **(Modified trichrome stain x 400)**


R7.GT:40
09


R14.GT:40X
08

matrix deposition around them which was visible as dark granular areas. Fibroblast proliferation state was studied by counting dark spots or areas of silver deposition (AgNOR count) of 100 nuclei in the peri-implant area under oil immersion objective of the microscope. The counting was done on 100 nuclei on each section and was expressed as a mean of 100 counts (Table 4). The AgNOR counts between different groups showed visibly appreciable differences (Fig. 15) and were highly significant statistically. In the control sections sham site could not be identified. However, AgNOR count of the resting fibrocytes in the endomysium was made (Fig. 16).

Table 4. AgNOR count of fibroblast around reactive and non-reactive biomaterial at seven and 14 days post implantation (n = 6)

Treatment groups AgNOR count (No)	Reactive biomaterial (Chromic catgut)		Non-reactive biomaterial (Polypropylene)		Control	
	7 days	14 days	7 days	14 days	7 days	14 days
Mean	1.97	2.23	1.81	1.67	1.33	1.36
SE	0.03	0.02	0.011	0.022	0.012	0.05

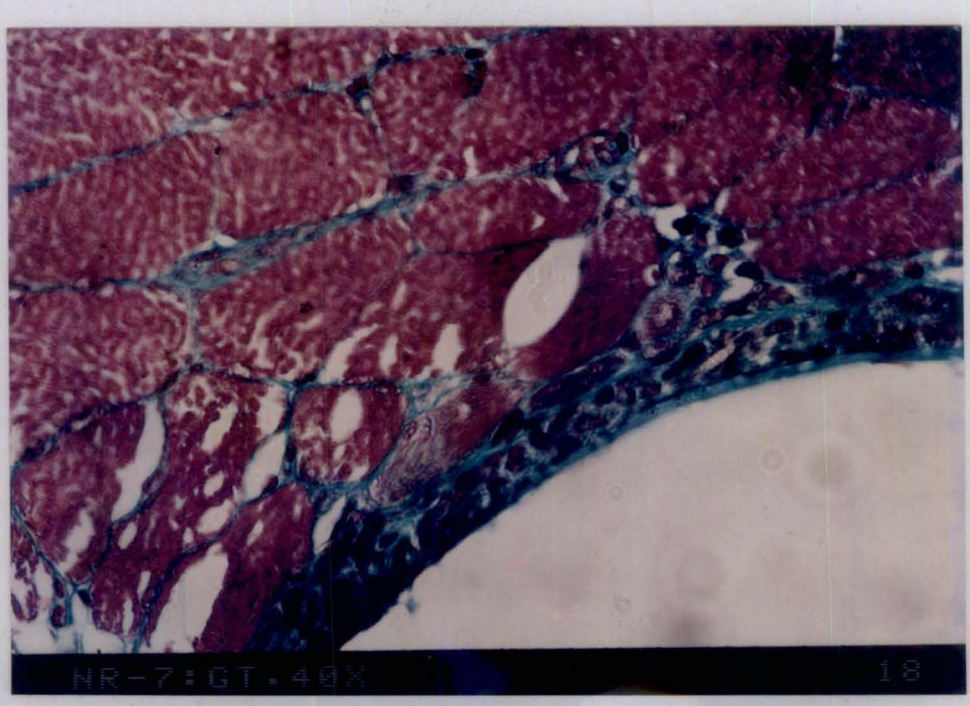
At seven days the AgNOR count around reactive biomaterial was 1.97 ±0.03 (Fig. 17). Sections showed mild granular deposits around the fibroblasts. At 14 days a higher fibroblast proliferative state was observed with an AgNOR count of 2.23 ±0.02. This section showed abundant peri-implant granular deposition around fibroblasts (Fig. 18).

The AgNOR count around non-reactive material at seven days was 1.81 ± 0.011. Section showed lesser peri-implant granular deposition (Fig. 19). At 14 days, sections showed even less AgNOR count (1.67± 0.02). Peri-implant granular deposition was the least compared to that around the reactive material (Fig. 20). Control section of seven days and 14 days showed AgNOR counts of 1.33 ± 0.012 and 1.36 ± 0.05 respectively. No granular deposition could be observed around the fibrocytes.

Analysis of data on AgNOR count showed statistically significant differences between groups on ANOVA test. Following this, 't' test for samples assuming equal / unequal variances were done among reactive and non-reactive groups and their respective controls based on F test results. Results revealed

Fig. 14 Non-reactive biomaterial at seven days post implantation: Mild fibrosis and mild collagen deposition **(Modified trichrome stain x 400)**

Fig. 16 Fibroblast proliferation response in control muscle tissue: Note the dark dots within the nucleus **(AgNOR stain x 1000)**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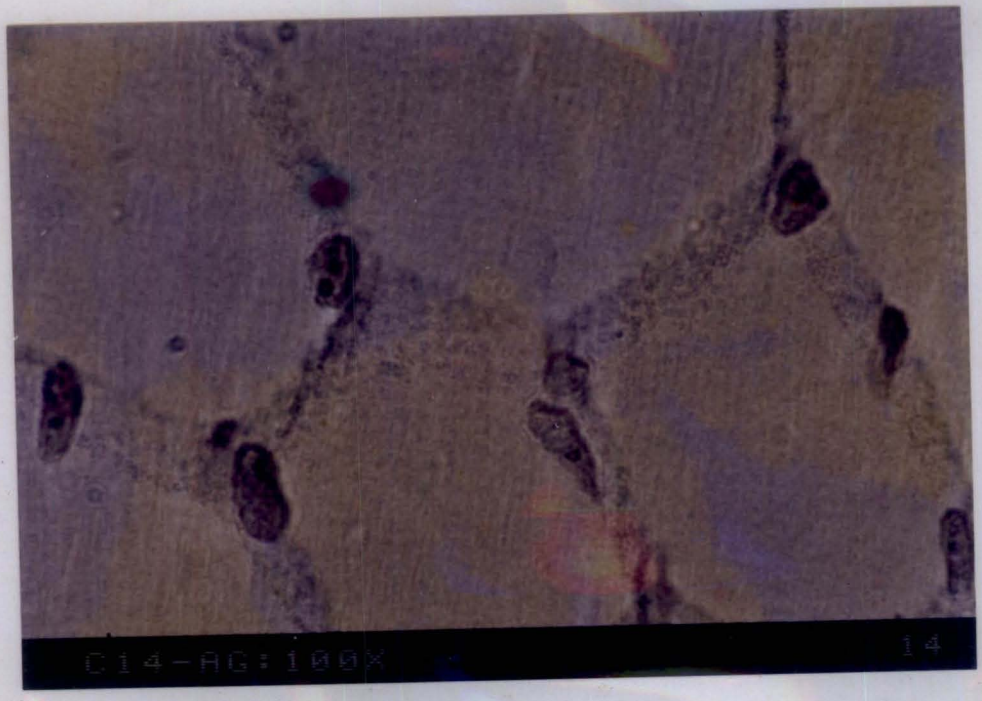
C14-AG:100X
14

statistically significant differences between each groups and also when compared to respective controls.

Most significant difference was noticed between control 14 days and reactive 14 days and the least significant difference was between non-reactive 7 days and non-reactive 14 days.

Fig. 15 AgNOR count of fibroblasts around reactive and non-reactive material at seven and 14 days post implantation (Mean of six samples with SE)

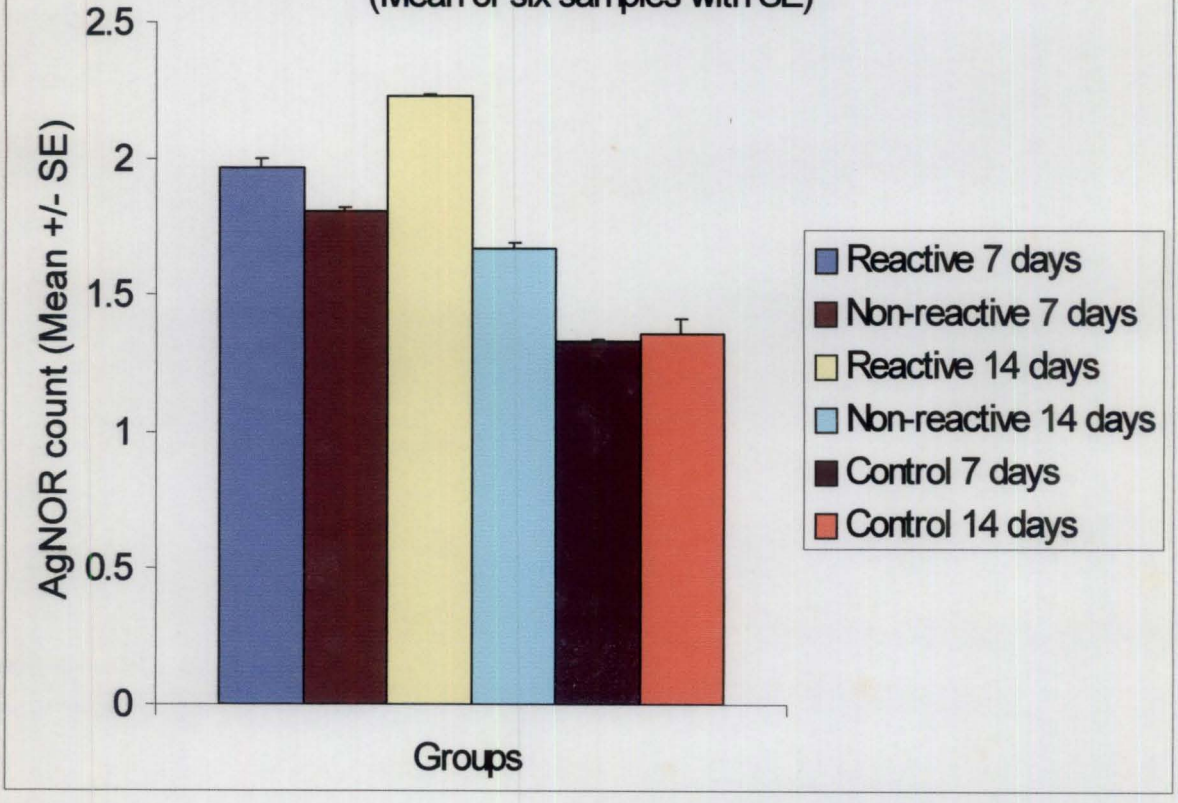

Fig. 17 Fibroblast proliferation response around reactive material seven days post implantation: Nucleolar organizer regions appeared as dark dots within the elliptical nucleus of fibroblasts. Mild granular deposits around fibroblasts noticed. **(AgNOR stain x 1000)**

Fig. 18 Fibroblast proliferation response around reactive material 14 days post implantation-: extra-cellular granular deposits around fibroblasts and presence of more AgNORs in the nucleus **(AgNOR stain x 1000).**


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12

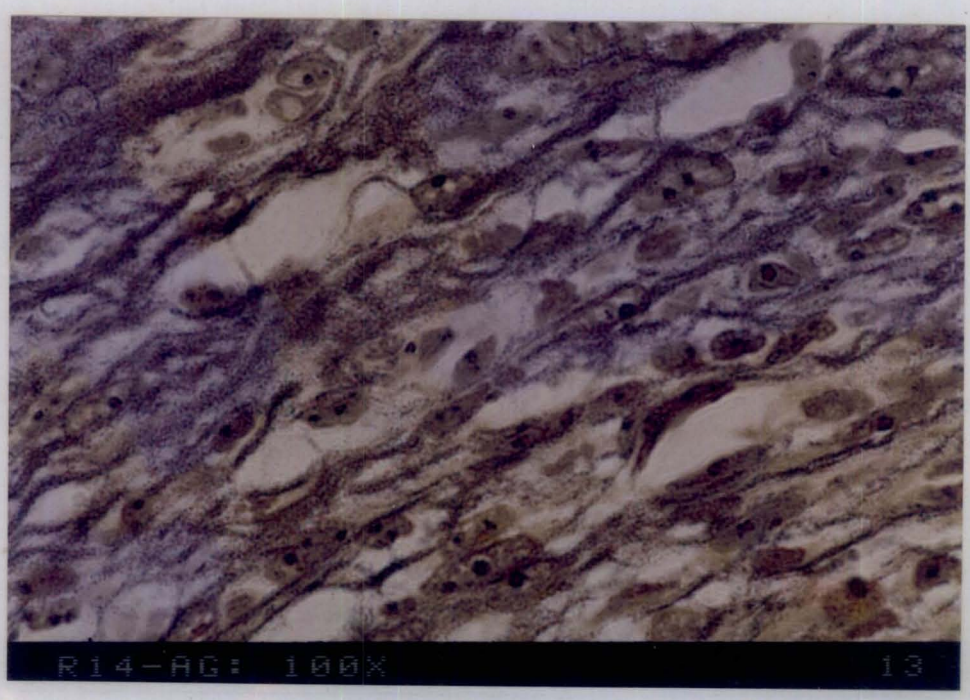
R14-AG: 100X
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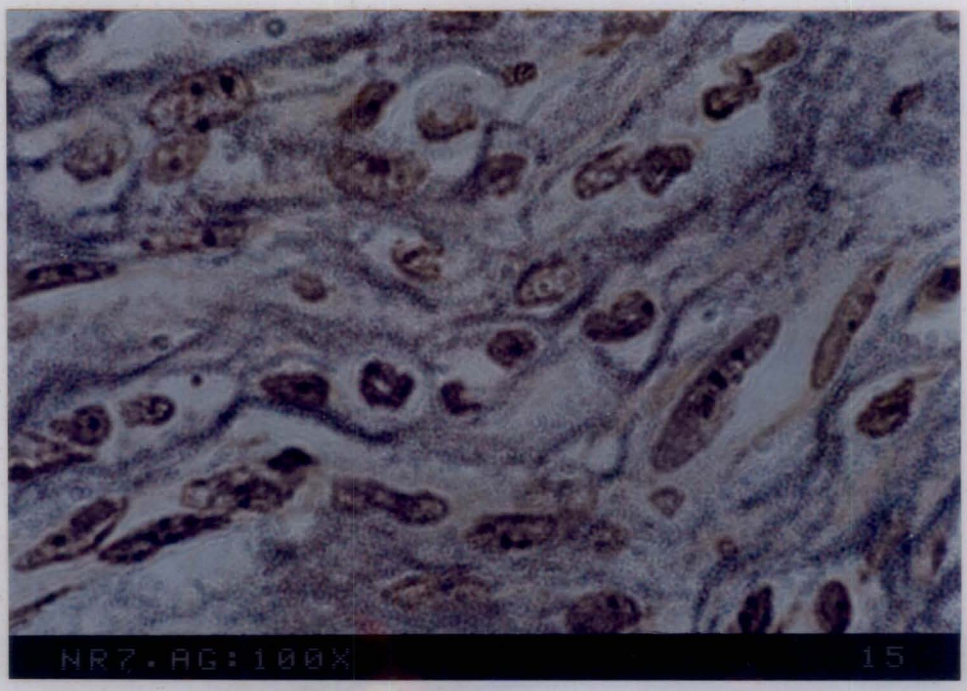
Fig. 19 Fibroblast proliferation response around non-reactive material seven days post implantation: Lesser extra-cellular granular deposits with two or less AgNORs in the nucleus **(AgNOR stain x 1000)**

Fig. 20 Fibroblast proliferation response around non-reactive material 14 days post implantation: Mild extra-cellular granular deposits with less AgNOR in the nucleus **(AgNOR stain x 1000)**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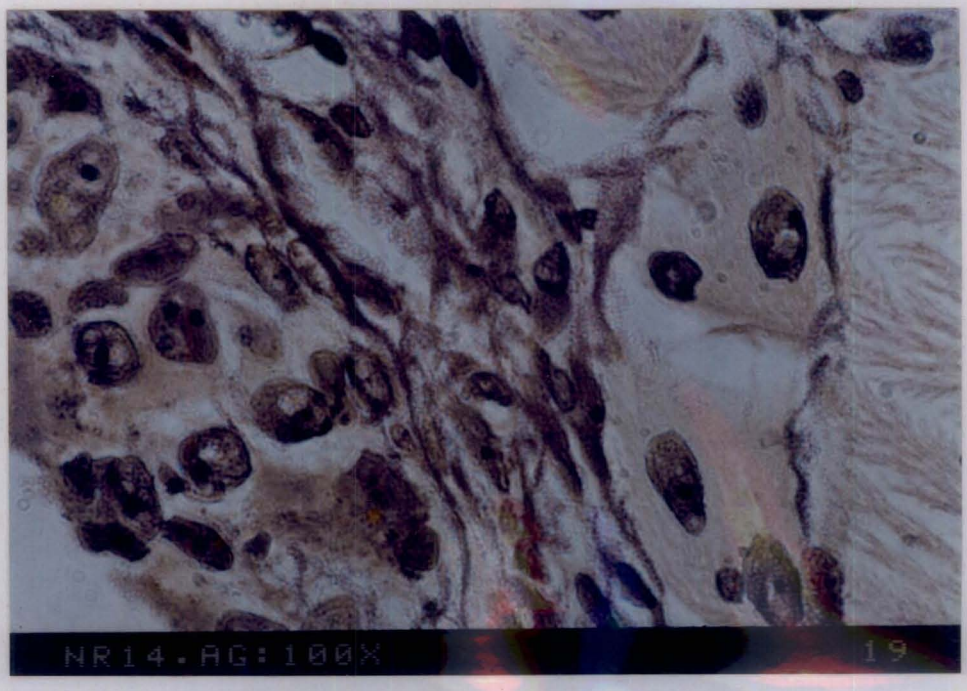
15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DISCUSSION

5 DISCUSSION

Biomaterials are used for the treatment of various surgical disease conditions both in man and animals. These materials that are intended for biomedical application are tested using a battery of standard tests both *in vitro* and *in vivo* before qualifying them as biomaterials. Among the tests used, short-term intramuscular implantation test is a screening method of toxicity testing for both the leachables as well as the biomaterial itself following implantation into an animal. It provides an opportunity to study the cellular responses to the material at the tissue-material interface, which is also used besides the results of other tests to predict the suitability of a material as biomaterial. However, it is still uncertain what molecular and cellular responses are critical in host-material interactions (Hunt *et al.*, 1996). Hence this study was envisaged with the purpose to record the proliferative response of fibroblasts using AgNOR technique in the peri-implant area of two different types of implants- (a). Reactive biomaterial (chromic catgut) and (b). Non-reactive biomaterial (polypropylene). These

materials in the form of sutures were selected following the recommendations of Gourlay *et al.* (1978) and Cholvin (1986). The study duration of seven and fourteen days were selected based on literature (Woodward and Salthouse, 1986) assuming optimum fibroblast proliferative activity during these periods. Addition of sham surgery group as controls in the experiment was to study the normal tissue response to surgical procedure without an implant. AgNOR technique was used to study the state of fibroblast proliferation. It can be applied on formalin fixed paraffin embedded tissue as used routinely and is less complex compared to other immuno-histochemical techniques. It also gives reasonably accurate results comparable to other immuno-histochemical techniques (Prakash *et al.*, 1997; Korkolopoulou *et al.*, 1998). Hematoxylin and eosin staining was used to observe a reactive and non-reactive tissue response. Collagen estimation in the implanted tissue and special stains for collagen were used to confirm fibrosis and collagen deposition (fibroblastic activity) around peri-implant area during reactive and non-reactive tissue response at seven and 14 days.

Collagen content of the implanted tissue between groups (reactive and non-reactive material) at both time duration showed no statistically significant difference. Wider error bars noticed in the estimation of means might have contributed to this. In collagen estimation approximately five milligrams of lyophilized tissue sample was used. On lyophilisation peri-implant area became less distinct from the surrounding muscle tissue making it difficult to accurately sample the area. The above two factors might have contributed for a wider error bar which finally resulted in statistically non-significant difference even when there was a visible difference between groups.

Histopathological evaluation using hematoxylin and eosin staining revealed a definitive picture of tissue response as reported in the literature (Woodward and Salthouse, 1986). The tissue response around the reactive material (chromic catgut) showed an initial tissue response of acute inflammation at seven days post implantation followed by a gradual transition to chronic response by 14 days post implantation as it was recorded elsewhere (Golden, 1982). At seven days post implantation the type of

cellular response seen was typical of acute inflammation characterised by infiltration of neutrophils and macrophages. Similar observations were noticed by Ryan and Majuo (1977) and Hurley (1983). Mild fibrosis and mild collagen deposition as observed in sections stained with special stains for collagen indicated the reduced fibroblastic activity during this period. At 14 days post implantation reactive biomaterial has incited a mild chronic type of inflammatory tissue response. Here fibroblasts in many layers could be observed in the peri-implant area by hematoxylin and eosin staining. Special stains for collagen demonstrated severe fibrosis with good collagen deposition. Literature also points to such a tissue response to reactive material such as surgical catgut at this duration (Gourlay *et al.*, 1978; Golden, 1982). Presence of hemosiderin laden macrophages in the peri-implant area signifies only old hemorrhages which occurred at the time of implantation. Muscle necrosis noticed could be attributed to the surgical injury itself as well as to the toxic character of implant or its leachables (Woodword and Salthouse, 1986). Hemorrhage is part of the inflammatory response observed. Fibrosis and neovascularisation are indicative of healing response from the tissue.

Non-reactive biomaterial (polypropylene) incited an acute inflammation at seven days post implantation with thicker reaction zone when compared to reactive seven days group. Similar observation has been made on polypropylene at shorter durations (Chu, 1983). Level of fibrosis and collagen deposition was minimal as observed in sections stained with special stains for collagen. The tissue response was comparable to that around reactive material at the same duration. This clearly demonstrated that by seven days post implantation, the tissue response was indistinguishable in both reactive and non-reactive materials. Interestingly scar thickness also showed no statistically significant difference between reactive and non-reactive biomaterial at seven days post implantation.

By 14 days post implantation there was a dramatic change in the tissue response against the non-reactive biomaterial. The tissue response observed was that of a mild chronic nature with extremely thin reaction zone. The scar thickness noted was also the least when compared to other groups. Special stains for collagen showed mild fibrosis and mild collagen deposition.

When compared to reactive material of the same duration even though the inflammatory response for both reactive and non-reactive biomaterial was mild chronic in nature, there was a profound difference in the level of fibrosis and collagen deposition between these two groups. The reactive material at 14 days post implantation incited severe fibrosis and good collagen deposition. Scar thickness was also significantly higher for reactive material at 14 days post implantation compared to non-reactive material of the same duration. Another interesting observation was that the tissue response to non-reactive biomaterial *i.e.* scar thickness at 14 days was similar to that observed at seven days post implantation. These observations demonstrated that at 14 days post implantation there was profound difference in tissue response between reactive and non-reactive material.

Sections stained well with the AgNOR staining technique used. Silver nitrate and gelatin in formic acid solutions were used up to two weeks after preparation and it stained well. All precautions as recommended by Newman and Jasani (1998) in the preparation and storage of all the reagents were followed to achieve this. After AgNOR staining,

fibroblasts could be identified by their elliptical nuclei and abundant extra cellular granular depositions under oil immersion objective, which might include collagen. AgNOR could be identified as brown to black spots in the nuclei. AgNOR staining technique could be applied with reasonable reproducibility in different sections of the same groups or in different groups as demonstrated by smaller error bars to the means.

On interpreting AgNOR counts in the fibroblast nuclei, sections of reactive material at seven days post implantation showed a fibroblastic proliferation state which was comparatively less than that was at 14 days post implantation. Reactive material at 14 days post implantation produced a severe fibroblastic proliferative response with an AgNOR count of 2.23±0.023 and an abundant extracellular granular deposition. Another interesting observation made was that certain fibroblast nuclei around reactive material at 14 days showed higher number of spots (upto seven) compared to less number (upto five) noted at seven days post implantation. This may be due to vigorous proliferative activity of fibroblast as indicated in literature by Bratulic *et al.* (1996),

around reactive implant at 14 days when compared to the seven days post implantation. Special stains for collagen in tissue around reactive implant also supported this finding by showing mild fibrosis and mild collagen deposition at seven days and severe fibrosis and good collagen deposition at 14 days post implantation. In addition scar thickness around the reactive implant showed a considerably thicker scar at 14 days compared to that of seven days post implantation.

Sections of tissue response around non-reactive material showed fibroblast in proliferative activity which was less, compared to that seen around reactive material at both seven days and 14 days post-implantation. Fourteen days post implantation samples showed minimal proliferative activity, which was only next to resting fibrocytes in the perimycium. Special stains for collagen have also depicted similar fibroblast response in these sections. Moreover the scar thickness observed around non-reactive implant at both durations of seven and 14 days also supported this finding.

The above observations on statistical comparison showed statistically significant difference between

groups. Fibroblasts around reactive material at 14 days post implantation showed highest proliferative activity followed by the proliferative activity around the reactive material at seven days. Least proliferative activity was observed in the control. These observations very well agree with the literature on cellular response against reactive material and non-reactive material at both the duration of seven and 14 days post implantation (Woodward and Salthouse, 1986). Control samples at seven and 14 days duration did not show any statistically significant difference between them. On P value comparison of data of fibroblastic proliferative activity with the AgNOR count on fibroblast cells in the muscle tissue of respective control groups, highest fibroblast proliferative activity around reactive material at 14 days post implantation was noted. Interestingly the difference between the fibroblast response around non-reactive material and its control at seven days duration came next. This might be due to the small error bar observed in the calculation of mean of non-reactive seven days group. Least significant difference in the fibroblast proliferative activity was observed between non-reactive 7 days and non-reactive 14 days. This supports the above findings.

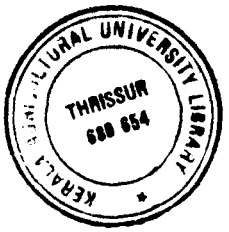
Values of scar thickness around implant and AgNOR count of fibroblast in the peri-implant area when plotted, illustrated similar trends and a correlation coefficient of 0.9 was obtained on their relation.

By using AgNOR technique of staining, a reactive material could be distinctly identified from a non-reactive material at both the duration of seven and 14 days post implantation. Routine hematoxylin and eosin staining failed to distinguish a reactive from non-reactive material at seven days post implantation by cellular response, although at 14 days they could be clearly differentiated. Even scar thickness measurement around reactive and non-reactive implant on Trichrome stained sections at seven days post implantation showed no statistically significant difference between them. Reactive and non-reactive materials could be distinctly identified at 14 days post implantation using AgNOR technique as well as other routine techniques tried here.

These observations clearly support AgNOR count test as a suitable method for *in vivo* evaluation of biomaterials by histopathological response. Evaluation of biocompatibility of a material by histological response alone established a difference only at 14

days while AgNOR count test demonstrated a difference as early as seven days. The AgNOR count test could be used as an early screening method. However, to decipher the type of inflammatory response/ cellular response incited by the implant, routine evaluation on hematoxylin and eosin stained sections are indispensable. The AgNOR count test for biocompatibility evaluation of a biomaterial standardized in this work can definitely be a valuable supplementary aid to the routine hematoxylin and eosin staining in the evaluation of biocompatibility of materials. More over AgNOR count technique might find an application in distinguishing a better biocompatible material out of biomaterials, which have proved biocompatible by other methods.

SUMMARY



6 SUMMARY

The study of AgNOR count technique for biomaterial evaluation was conducted at the Department of Pathology, College of Veterinary and Animal Sciences, Mannuthy and Biomedical Technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram during 1999-2001. The efficacy of AgNOR count test was evaluated by comparing it with a routine method of biomaterial evaluation using short-term intramuscular implantation in the rat gluteus muscle. Following intramuscular implantation of reactive and non-reactive biomaterial for seven and 14 days duration, the cellular response to the material was studied using hematoxylin -eosin stained sections. Tissue response like scar thickness, fibrosis and collagen deposition was studied using sections stained with special stains for collagen. Besides this collagen content of the reactive and non-reactive material implanted for 7 and 14 days duration and in the sham operated muscle was determined

biochemically. Simultaneously fibroblast proliferation state around the reactive and non-reactive implant at 7 seven and 14 days was studied using AgNOR technique.

AgNOR technique was found to be sensitive in distinguishing reactive and non-reactive biomaterial even at seven days post implantation. Whereas the routine technique using hematoxylin-eosin staining or scar thickness estimation following special staining for collagen could not differentiate reactive and non-reactive sample at this duration. At 14 days post implantation AgNOR technique showed remarkable difference between reactive and non-reactive material. Similar observation was made in the routine technique also. Estimation of collagen around reactive and non-reactive material implanted muscle and in sham operated controls did not show any difference among them. With the technique adopted in this work for AgNOR staining, sections could be stained with good clarity and reproducibility which enabled accurate interpretation on them.

AgNOR count test can be reliably used for *in vivo* biomaterial evaluation even at seven days. The AgNOR count test of biomaterial evaluation proposed and

standardized in this work can definitely be a valuable supplementary aid to the routine hematoxylin and eosin staining in the evaluation of biocompatibility of materials.

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Appendix

Format for recording histopathological observations:

Name of the investigator: Dr. P.R. Umashankar

Date:

Animals number			
Duration of implantation			
Group			
Staining method			
Grade of observation	Mild	Moderate	Severe
Necrosis			
Degeneration			
Inflammation Hemorrhage			
Neutrophils			
Lymphocytes			
Eosinophils			
Plasma cells			
Macrophages			
Fibrosis			
Giant cells			
Foreign body debris			
Fatty infiltration			
Scar thickness			
Remarks:			

EFFICACY OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGION COUNT TEST FOR *in vivo* BIOMATERIAL EVALUATION

By

P. R. UMASHANKAR

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree of

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2001

ABSTRACT

The efficacy of Argyrophilic Nucleolar Organizer Region (AgNOR) Count Test in the evaluation of biomaterial was assessed in this study utilising a reactive (medium chromic catgut) and a non-reactive (polypropylene) material.

The materials were implanted into rat gluteus muscle for a duration of seven and 14 days and sham surgery done on a separate group of animals served as the control.

The tissue responses such as inflammation, muscle degeneration and peri-implant scar thickness around the reactive and non-reactive implant and the controls at seven and 14 days were studied using H&E and special stains for collagen. Besides this the estimation of collagen of the reactive and non-reactive material implanted tissue was studied. The observations made in this were compared with the fibroblast proliferation response at the respective durations as assessed by the AgNOR count estimation.

On histopathological evaluation, the reactive material showed an initial acute inflammatory response with mild fibrosis and collagen deposition which subsequently settled

into a chronic form with severe fibrosis and more collagen deposition at the end of 14 days. Non-reactive material elicited an acute inflammation initially (seven days post implantation) which settled into a mild chronic response at 14 days post implantation. Mild fibrosis and less collagen deposition were noticed in the peri-implant area at both the duration. Observations on fibroblast proliferation as assessed by AgNOR count test well correlated to the above findings. The AgNOR counts observed with reactive material at seven days and 14 days were 1.97 ± 0.03 and 2.23 ± 0.02 respectively. The counts for non-reactive material were 1.81 ± 0.01 and 1.67 ± 0.02 at seven and 14 days post implantation respectively.

The collagen content of the reactive and non-reactive implanted tissue did not reveal any statistically significant difference as compared with the respective control. From this investigation it was proved that AgNOR count test could be reliably applied for biomaterial evaluation even at seven days duration and it formed a valuable adjunct to the routine method employed in the *in vivo* evaluation of biomaterials.