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VARIATIONS
in
SILVER STAINED NUCLEOLAR ORGANISER
REGIONS (AgNORs)
in NORMAL, IRRADIATED and NEOPLASTIC
TISSUES

Volume I

By
Joseph Gallagher McDermott

Thesis submitted for the Degree of Doctor of Philosophy

Faculty of Science
Trinity College Dublin
July 2000

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TISSUES

Volume 1



Thesis
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Joseph Gallagher M.D.Sc.

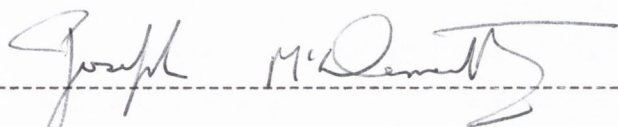
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DECLARATION

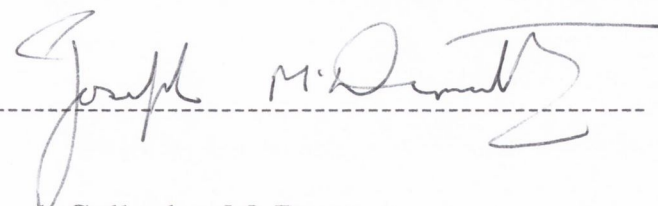
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SUMMARY

Nucleolar organiser regions (NORs) are loops of ribosomal DNA which contain the genes that transcribe to ribosomal RNA. They are situated on the short arm of the acrocentric human chromosomes 13, 14, 15, 21 and 22. Several of their associated nucleolar organiser region proteins, including Nucleolin (C23) and Numatrin (B23) can be demonstrated as tiny black dots contained within the nucleus by silver-staining (AgNOR technique).

In the current research the AgNOR technique was used to assess variations in the mean AgNOR area/nucleus in control and irradiated mouse small intestine (jejunum).

An investigation was then carried out to determine if there was a relationship between mean AgNOR area/nucleus and mean AgNOR number/nucleus, post-irradiation. Finally the scanning electron microscope was used to observe nucleolar organiser region associated proteins in normal and irradiated tissues. Although the main theme of the study related to variations in silver-stained nucleolar organiser regions in control and irradiated tissues, neoplastic NORs were also included in the scanning electron microscope section as an interesting addendum to the research.

In control tissues the mean AgNOR area/nucleus ranged from $0.44\mu\text{m}^2$ in the upper villus to $1.2\mu\text{m}^2$ in the cryptal epithelium. In post-irradiation samples the mean AgNOR area/nucleus within the cryptal epithelium increased significantly. The increase in mean AgNOR area/nucleus within proliferating cells of the cryptal epithelium may be due to radiation induced changes in the molecular structure of the NOR associated proteins. This increase was not present in the other compartments under review.

There was a inverse relationship within the cryptal epithelium compartment, post-irradiation, between the mean AgNOR area/nucleus and the mean AgNOR number/nucleus, i.e. as the AgNOR area

increased the AgNOR number decreased. The decrease in AgNOR numbers is thought to be due to a radiation induced inhibition of cell proliferation and the increase in AgNOR area may be caused by radiation induced changes to NOR associated protein structure. This phenomenon was not apparent in any of the other compartments.

The use of the scanning electron microscope demonstrated that tumour AgNORs unlike those of control or irradiated samples, were bizarre "iceberg like" structures with a markedly increased surface area.

The study concluded that the measurement of the mean AgNOR area/nucleus using a light microscope may be an additional factor for assessing radiation damage within the small intestine. The study also suggested that in tumour pathology measurement of AgNOR area/nucleus using the light microscope may be of limited value. However using the AgNOR technique in combination with the scanning electron microscope may be of value in providing additional information regarding prognosis.

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ABBREVIATIONS AND SYMBOLS

AgNOR	- Silver-Stained Nucleolar Organiser Region
ANOVA	- One Way Analysis of Variance
B23	- Numatrin
C23	- Nucleolin
Co	- Cobalt
FiNORs	- Fluorescent Staining Nucleolar Organiser Regions
Gy	- Gray
HPFs	- High Power Fields
MeV	- Milli Electron Volts
Mol	- Morphological Index
NOR	- Nucleolar Organiser Region
NORAP	- Nucleolar Organiser Region Associated Protein
PCNA	- Proliferative Cell Nuclear Antigen
SEI	- Secondary Electron Immision
SEM	- Scanning electron microscope
-S-H	- Sulphydryl
-S-S	- Disulphide
TLI	- Thymidine Labelling Index

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Part 1.

INTRODUCTION

1. GENERAL INTRODUCTION

Over the past two decades the silver staining nucleolar organiser region (AgNOR) technique has been used to assess cell proliferation in tumour tissues. It has been used with varying degrees of success in order to determine grade of tumour and prognosis.

In this study the mean AgNOR area/nucleus was measured and compared within the various compartments of control mouse small intestine (jejunum). An assessment was then carried out of variations in mean AgNOR area/nucleus, post-irradiation. Doses ranged from 5Gy to 20Gy and post-irradiation time intervals from 30 minutes to 72 hours. Results of the mean AgNOR area/nucleus from this study were then compared with the results of mean AgNOR number/nucleus from previous research in order to investigate the possibility of a relationship between the two parameters. Finally the scanning electron microscope (standard emission & back-scattered mode) was used to observe the structure of AgNORs in control, irradiated and tumour tissues.

Before proceeding with this work it was necessary to examine the structure of the small intestine and to review radiation and its effects on cell structure. Applications of the AgNOR technique were studied with comment on other methods of assessing cellular proliferation.

2. STRUCTURE of the SMALL INTESTINE

Anatomically the small intestine is divided into three parts. The duodenum, jejunum and ileum. The surface area of the small intestine is enhanced by three factors that are crucial to the gut. These are the *plicae circulares*, the villi and the microvilli.

The *plicae circulares* are circular folds which consist of mucosal/submucosal invaginations located almost entirely in the duodenum and jejunum. They are visible on gross inspection. The *intestinal villi* are finger-like, tongue-like or leaf-shaped structures, depending on species, attached to the plicae that protrude into the intestinal lumen. They can be viewed under low-power microscopy and consist of a layer of epithelial cells overlying the lamina propria. Jejunal villi are tall and thin and ileal villi are short and broad. Finally the *microvilli* are tubular projections of the apical membrane of enterocytes that make up the epithelium.

The small intestine is composed of four concentric layers. From the lumen outwards they are the *mucosa*, *submucosa*, *muscularis externa* and the *serosa adventitia*.

Mucosa

The small intestinal mucosa consists of the *epithelial covering*, the *lamina propria* and the *muscularis mucosae*.

Epithelial Covering

The epithelium is a continuous sheet of simple columnar cells which rests on a basement membrane, overlying the villi and forming the crypts. It is the major barrier between the intestinal lumen and the lamina propria. The epithelial cells on the villi transport the products of digestion into the lamina propria where they enter the lymphatic and circulatory systems and are transported to other areas of the body. Tight junctions bind adjacent

epithelial cells together. This restricts fluxes between adjoining cells to small ions, small molecules and water.

The crypt and villus form the basic functional and structural unit of the small intestine. Stem cells located in the crypts of Lieberkuhn are the source of the four major terminally differentiated epithelial cell types. These are the enterocyte, the goblet cell, the enteroendocrine cell and the Paneth cell. Cellular differentiation proceeds during a complex process. The gut stem cell, postulated to be in the lower crypt region (Cheng et al 1972) gives rise to proliferating cells which differentiate as they migrate up the crypt on to the villus to become enterocytes, goblet cells and endocrine cells. Epithelial cell migration and differentiation occurs continuously and the process of cellular renewal takes approximately 3-5 days.

The Lamina Propria

The connective tissue core of the villus is known as the lamina propria. The immune cellular component includes lymphocytes, macrophages, granulocytes, plasma cells and mast cells. Also present in the lamina propria are the blood vessels some smooth muscle and fibroblasts.

Muscularis Mucosae

This is composed of a layer of smooth muscle which separates the mucosa from the submucosa.

The Submucosa

The submucosa is composed of a more solid connective tissue containing collagen, elastic fibres, fibroblasts and fat cells. In the duodenum there are specialised Brunner's glands that secrete mucus and bicarbonate. These are branched epithelial glandular structures that fill the submucosa and are thought to be important in neutralisation of stomach acid. The submucosa also contains ganglion cells and nerve fibres termed Meissner's plexus, a collection of nerves that communicate with Auerbach's plexus in the muscularis externa. These plexes interact to regulate and

epithelial cells together. This restricts fluxes between adjoining cells to small ions, small molecules and water.

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coordinate gut peristalsis.

The Muscularis Externa

The muscularis externa makes up the bulk of the intestinal wall. It is composed of smooth muscle arranged in two layers, i.e. the outer longitudinally orientated layer and the inner circular one. Between the two layers lies the Auerbach's nerve plexus (myenteric plexus), composed of ganglion cell bodies and nerve fibres. The muscular layers of the gut are responsible for generating coordinated peristaltic movements.

The Serosa Adventitia

The thin serosal lining of the small bowel consists of mesothelial cells overlying loose connective tissue. This contains collagenous and elastic fibres (Neutra and Padykula 1983).

Types of Small Intestinal Epithelial Cells

The small intestinal epithelium consists of columnar absorptive cells (*enterocytes*) interspaced by *goblet* cells. A few *enteroendocrine* cells are also present. *Paneth* cells are found only in the crypts. These four types of cells originate from gut epithelial stem cell, thought to be in the lower crypt region.

The Enterocyte

When cells migrate from the lower to the upper crypt, cellular differentiation begins. The cells that become absorptive enterocytes are the most abundant and important of the epithelial cells. They express a variety of specific genes which enable these cells to digest and absorb many different nutrients. The nucleus of the enterocyte is centrally located and shows dense masses of chromatin along its nuclear envelope (Cheng et al 1974). The surface of this cell is highly specialised for absorption by its covering of numerous microvilli.

The Goblet Cell

Mucous secreting goblet cells are present throughout the entire gastro intestinal tract but they are more numerous in the ileum than in the

jejunum. These cells exhibit a “brandy goblet” shape and are characterised by granules filled with mucin which acts as a lubricant within the gastro intestinal tract.

Enteroendocrine Cell

Endocrine cells are small pyramidal cells showing a clear cytoplasm, a pale spherical nucleus and dense granules in the basal region. These cells are classically characterised as argentaffin or argyrophil cells depending on their reaction to silver-staining in the presence or absence of a reducing agent.

Paneth Cell

Paneth cells are pyramid shaped cells that are thought to be important in the immune defensive role of the gut. They reside in the crypt base and contain large eosinophilic secretory granules. The renewal rate of Paneth cells is approximately 30 days which is slower than the rate of 5 days in the case of the mucous and absorptive cells. Their role in host defence has been suggested by their abundant expression of lysozyme (Peeters et al 1975) and their ability to degranulate in response to live and heat-killed bacteria (Sato et al 1986). Numerous studies have shown that Paneth cells produce many proteins (Defensins) related to this putative function. In particular Defensin 5 and Defensin 6 are specific to the small intestine. These peptides are 30-35 amino acids in length (Jones and Bevins 1993).

3. RADIATION

The term radiation is defined as a physical phenomenon in which energy travels through space, even though that space may otherwise be devoid of matter. There are two types of radiations.

Electromagnetic radiation consisting of magnetic or electrical disturbances, the exact level of the energy determining its wavelength. Electromagnetic radiations include radio waves, infra-red, visible light, ultra-violet, X and Gamma radiations. Only the last two are ionising radiations. Ionisation is defined as follows: if the incident particle completely removes an orbital electron from an atom then ionisation has occurred.

Particulate radiation consisting of atomic or subatomic particles, moving at high velocities. These particles include Beta particles (electrons) protons and neutrons.

Most radiations in and near the visible part of the spectrum form a major source of energy essential for biological processes. Radiations such as radio waves, microwaves, sunlight and infra-red waves are relatively harmless in normal amounts. High energy radiations whether electromagnetic or corpuscular can have a detrimental effect on biological systems.

Historical Introduction of Radiation, Radioactivity and Radioactive Substances

Ionising radiations were first discovered in 1895 when Roentgen was studying the properties of electrical discharge through cathode-ray tubes. He discovered a new type of radiation which he called X-rays. The earliest recognition of radioactivity was by Becquerel (1896), who noticed the chemical action of rays from uranium. Pierre and Marie Curie discovered the extreme activity of the ore of uranium (pitchblende). They

subsequently isolated a new element called radium. This was the first isolation of a naturally occurring radioactive element. From this work it was eventually shown that radioactivity occurs from the instability of the nucleus of the atom which gains stability by the release of particulate or electromagnetic radiations. The initial “miracle cure” for skin cancers and other superficial tumours using these crude radiation techniques led to great optimism. This was soon followed by a period where a lack of understanding of the physics and biological effects of radiation, the non-existence of dose measurement and primitive equipment led to “The Dark Ages” of radiation therapy (Kaplan 1979). The fact that exposure to ionising radiation produced hazardous effects on the gastrointestinal tract was recognised only one year after the discovery of X-rays (Walsh 1897).

Radiation therapy would soon have disappeared had it not been for the work carried out by Regaud at the Foundation Curie Laboratory, Paris. In 1919 Regaud began a classical series of experiments which showed that spermatogenesis in the testes could be permanently eradicated by the administration of successive daily doses of fractionated radiation. No single massive dose would produce the same result without inflicting permanent and often intolerable injury to the overlying skin (Regaud 1922).

On the hypothesis that the testes, with its high rate of cell turnover, might represent a model for a malignant tumour. Regaud suggested that the fractionated radiotherapy technique could be used on malignant neoplasms. Regaud and his colleague Coutard applied these revolutionary techniques to a variety of cancers of the head and neck region. Impressive results also appeared for treatment of cervical cancers. Accordingly fractionation of treatment was developed into the universally accepted techniques of present day radiotherapy.

In 1913 Coolidge advanced the technology of radiation therapy. He invented a vacuum X-ray tube which could operate at the extremely high energy of 200kV. This period, known as the “kilovolt era”, was a period of

great success in the treatment of superficial cancers (Busch 1970). Following World War II new devices to increase the voltage from Kilovolts to Megavolts were developed. This period became known as the "Megavoltage Era". New inventions (the betatron and the Linear Electron Accelerator) produced beams of very high energy while working at low voltages (Kaplan 1979). Many other radiation sources and techniques are now available including Cobalt Teletherapy apparatus capable of providing external gamma rays. Other useful techniques are interstitial implantation and intracavitary placement of needles, capsules, tubes, threads and other devices containing natural and artificial radioactive isotopes used for highly localised deposition of ionising radiation in various deep tissues within the body. A variety of particulate radiations including electrons, protons, neutrons and heavy ions are also used in the field of radiotherapy.

Current Role of Radiation Therapy

The availability of new devices and techniques has made a major contribution to the precision of modern radiation therapy. Radiotherapy at present occupies an important role in the treatment of most cancers, usually being used as a second line treatment following surgery. It is also being used to reduce tumour size prior to primary treatment. This phenomenon has changed the clinical approach for Hodgkin's disease and other malignant lymphomas (Goffinet et al 1976). There have also been improvements in the long term survival rates for several types of deep seated tumours. These include lymphomas and those tumours arising in the pelvis, the head and neck regions.

Measuring Radiation

The amount or quantity of radiation measured in terms of the energy absorbed in the tissues is called the dose. The first unit of radiation was named the Roentgen after the discoverer of the X-ray. This was reported by Siegbahn in 1928. The Roentgen is defined as "That amount of X or Gamma radiation such that the associated corpuscular emission

per 0.001293 gramme of air, produces in air ions carrying one electrostatic unit of charge of either sign" (Meredith and Massey 1977). Refinement of dose values in 1956 led to the unit of absorbed dose, the rad (Kaplan 1977). One rad is a dose which equals an absorption of 100 ergs per gram of substance irradiated (Meredith and Massey 1977).

In 1962 it was generally accepted that the Roentgen should be the unit of exposure and the rad the unit of absorbed dose (Meredith and Massey 1977). The old unit of absorbed dose has been replaced by the Gray (Gy) which is equivalent to 100 rads (Meredith and Massey 1977). The centiGray (1cGy = 1 rad) has also come into common usage.

Effects of Irradiation on Cells and Deoxyribonucleic Acid (DNA)

The biological targets of radiation are the cells which make up the body's various tissues. It is the interaction of radiation with the cell that starts a chain of molecular events that result ultimately in the inhibition of the cell division. The exact mechanism by which the cells reproductive capability is damaged remains unknown. The specific target of radiation damage is the deoxyribonucleic acid (DNA) molecule. The two spiral threads of DNA twist around each other to form the double helix. DNA is composed of purine and pyrimidine compounds known as bases, and of phosphate and sugar molecules. The paired purine bases are adenine and guanine. The pyrimidine bases are cytosine and thymine. DNA is composed of two complementary strands. Radiation may produce single or double strand breaks within DNA. Single strand breaks are more amenable to cellular repair. However double strand breaks are more difficult to repair and can irreversibly damage the cell. Cells may go through a few divisions before dying. Radiation damage may result in changes (mutations) in the structure of the DNA. Radiations may also result in breaks appearing in the chromosomes. Broken ends of different chromosomes may rejoin. This is called a chromosomal rearrangement. In chromosomal rearrangements it is

the double strand breaks in DNA that are thought to be critical. The evidence of chromosomal abnormalities increases with the dose of ionising radiation.

Radiation has both direct and indirect effects at a molecular level. The *direct action* is the ionisation of atoms in the cell nucleus. The definition of ionisation is the removal of an electron from one of the outer shells of an atom. The *indirect action* is much more important in radiotherapy. When X-rays interact with water, free radical ions are produced. The H_2O^+ is chemically unstable and quickly forms a hydrogen ion⁺ and a hydroxyl ion (OH^+). These free radicals are highly reactive and result in breaks in the chromosomes. Much of this damage is repaired, i.e. rejoining of the broken chromosomes. Immediate cell death may result after massive radiation doses (e.g. 1000Gy from an atomic bomb), however, at lower dose levels, as used in clinical radiotherapy, the chromosome damage does not reveal itself until the cell attempts to go into mitosis.

Response of Small Intestine to Radiation Injury

The response of the small intestine to radiation injury has been studied in various animal models (mice, rats, cats etc) including humans using a variety of types of radiation. These studies have been permuted with whole body, partial body and intestinal radiations at various doses and time points. Because of the wide variations in this previous work, it is difficult to obtain a coherent picture of radiation-induced changes in the small intestine.

Two types of microscopy will be reviewed (i) light microscopy and (ii) scanning electron microscopy.

(i) Light Microscopy

In order to gain a better understanding of the complicated chain of reactions that takes place after irradiation of tissues a data display was introduced that could be referred to when assessing total radiation damage

(Carr et al 1992). This study showed quantitative comparisons by individual cell types. The response to 5Gy, 10Gy, and 20Gy was sampled three days after treatment and the 10Gy sample 6 hours, 1 day and 3 days after treatment. This analysis has quantified fourteen separate cell types and structures including crypt numbers and mitotic figures. Tissue percentage and tissue area were also established for epithelium, stroma, muscle and nerve tissue. This data was characterised with a formula known as the Morphological Index (MoI). The Morphological Index has given a more defined expression of radiation damage. Sham specimens also showed some damage. It was shown that even the stress of being slightly sedated by ether and restrained in a jig has caused damage to the duodenum. This approach shows a comparison to existing standard histopathological methods in that this study is quantitative rather than the review of ulceration, vascular distortion and inflammation.

Four years later, an investigation was carried out to determine histological changes in tissues following irradiation of the small intestine of rats (Rubio et al 1996). In this research the distal ileum was irradiated with low doses (9-12Gy), moderate doses (15-18Gy) and high doses (21-24Gy) of radiation. The animals were sacrificed after 3, 10 and 30 days. A total of 16 histological parameters were found to be relevant. These included, cell necrosis, loss of goblet cells, crypt abscesses, structural changes of the crypts, loss of margination of lymphocytes, increased collagen in the submucosa and mucularis ulcerations. These parameters were found to be dose and time dependent. Racket-shaped superficial epithelial cells, capillary congestion and an increased number of round cells in the lamina propria were found to be only time dependent histological parameters.

Six months later a study was carried out to investigate changes in the cell kinetics in rat small intestine after gamma ray exposure at different times of the day (Becciolini et al 1996). Morphological changes in rat jejunum were studied after whole-body exposure to 3Gy of gamma rays.

The different times of the day were midnight, 0600, 1200 and 1800 hours. The number of epithelial cells and mitotic indices were evaluated. After 12 hours there was a marked reduction of all parameters, but the proliferative activity was restored quickly and at 36 hours after irradiation the values were higher than controls. The authors concluded that the animals irradiated at different times of the day showed a similar general post-irradiation response.

The Morphological Index (MoI) (Carr et al 1992) has also been used to determine the response of mouse small intestine to X-irradiation and neutron irradiation (Carr et al 1996). This study concluded that neutron treatment led to more damage to the neuromuscular components of the small intestinal wall, while X-irradiation produced early vascular changes.

(ii) Scanning Electron Microscopy

The effects of radiation-induced changes on the surface morphology of intestinal villi have been found to be dependent on dose and type of radiation (Carr and Toner 1972). This work described the effect of supra-lethal doses of radiation (15-25Gy) on intestinal villi after whole body X-irradiation. Forty-eight hours post irradiation the villus morphology appeared unaltered. Progressive atrophic changes were seen from 48-100 hours after irradiation. The period from 48-70 hours resulted in the intestinal villi appearing distorted and clumped. Around 70 hours the villi appeared shorter, conical and stunted. At around 90-100 hours after irradiation some villi appear as stunted projections. At this stage surface creases disappeared and irregular projections were seen on the surface of the villi. Rat intestinal villi had a similar morphological effect when treated with whole body irradiation (Anderson and Withers 1973).

A quantitative assessment of villus damage was proposed by Carr et al (1983), who devised a scoring system based on ratio of drop in villus area. This system used a grid to divide the surface into squares. This was termed the single score method. Damage to mouse villi was assessed, using

the grid score method, in a period of 0-5 days after radiation with X-rays or neutrons. The results from the grid score system showed that damage to the villi was greater after neutron irradiation than following X-irradiation.

4. ASSESSMENT of CELL PROLIFERATION

Cell proliferation is one of the most fundamental biological processes. The assessment of cell proliferation is based on the interpretation of features of the microscopic image. In recent years there has evolved an increased interest in processing quantifiable data from these images. Before reviewing techniques of measuring cell proliferation it is necessary to mention the cell cycle.

The Cell Cycle

Following mitosis, the daughter cells enter the GAP 1 (G1) phase in which they spend a period of time which can vary depending on the type of tissue. They then enter the S phase where the cell's genetic material is doubled during DNA synthesis.

Following this the cells move into a second GAP phase (G2) before the cells divide again. The time between two mitoses is called *The Cell Cycle Time*. This varies depending on the duration of the G1 phase.

Methods of Assessing Cellular Proliferation

Mitotic Figure Counting

The mitotic count was the first method which pathologists used to evaluate neoplastic cell proliferative activity. The mitotic count is usually carried out by tabulating the total number of mitotic figures in 10 High Power Fields (x40) of haematoxylin/eosin histological sections. Even though with some tumours the mitotic count has been significantly correlated to patient survival (Akerman et al 1987; Taylor et al 1966) the use of this parameter in routine histopathology is not recommended. Pyknotic and highly basophilic nuclei are not always easily distinguishable from mitosis. The definition of the number of mitotic figures is therefore subjective and remains controversial. Wide variations between observers have been reported (Silverberg et al 1976). In this study concern was expressed about the uses of mitotic counts as the sole indicator of malignancy.

Thymidine Labelling Index

The introduction of tritiated thymidine and the development of high resolution autoradiography during the 1950's led to the use of thymidine as the "gold standard" for cell kinetics. The thymidine labelling index (TLI) is a measure of the percentage of tumour cells in the DNA synthetic (S) phase of the cell cycle.

Bromodeoxyuridine

The S-phase labelling index can also be measured by using bromodeoxyuridine. This pyrimidine analogue is readily incorporated into cells during the DNA synthetic phase. This molecule can be detected by specific antibodies either in tissue sections or by flow cytometry. Tumours which have been exposed to bromodeoxyuridine, *in vivo* or *in vitro* are stained with the antibody. The stained cells are counted using phase microscopy and expressed as a percentage of 500 or 1,000 tumour cells to yield a labelling index.

Flow Cytometry

The capacity of some dyes to bind to DNA in a stoichiometric manner means that the amount of DNA present in the nucleus can be determined. The procedure involves the examination of a single cell suspension passing a given point by a suitable tuned laser beam, with reflected or transmitted light, or both, being detected and converted into an electronic signal. The presence on the cell of a fluorescent dye linked to an antibody or fluorescence due to a dye bound to nucleic acid can be detected. Information about the number of cells and their DNA content can be obtained from this data.

Immuno-histochemical Methods

Immuno-histochemical identification of nuclear antigens associated with cellular proliferation is another means of understanding this process. A particular advantage of immuno-histochemical demonstration of cell cycle related antigens allows spatial orientation to be shown. The mouse

monoclonal antibody Ki-67 identifies a poorly characterised nuclear antigen associated with the cell cycle, being expressed in all phases except G₀ (Gerdes et al 1983). Several reviews have shown that there is a close correlation between Ki-67 immunoreactivity and other variables of cellular proliferation such as thymidine labelling (Silvestrini et al 1989; Kamel et al 1989) in tumour tissues. Developments of the Ki-67 antibody, ie: Ki-67 clone MIB1 (Gerdes et al 1992), have demonstrated that this immunochemistry technique is a reliable and convenient method of assessing cellular proliferation.

Proliferating Cell Nuclear Antigen (PCNA) is another important cellular protein which is thought to be directly or indirectly involved with the control of cellular proliferation. PCNA functions as a co-factor DNA polymerase and is essential for completion of the cell cycle. PCNA antibody reacts with proliferating cells in a wide range of normal tissues, but the proportion of proliferating cells with PCNA immunoreactivity remains uncertain at present.

Silver-Stained Nucleolar Organiser Regions

The final method for review of cellular proliferation assessment is the AgNOR technique. This will be discussed in the next section.

5. NUCLEOLAR ORGANISER REGIONS

Nucleoli consist of three substructures. These are the fibrillar centre, dense fibrillar and granular components. The fibrillar centre is the specific region where the ribosomal (rRNA) transcript is generated and is considered to be the nucleolar organiser region.

The nucleolar organiser regions (NORs) are loops of ribosomal DNA (rDNA) which contain the genes which transcribe to ribosomal RNA (rRNA). NORs reside on the short arm of the acrocentric human chromosomes 13, 14, 15, 21 and 22 and have been identified using in situ hybridisation with radiolabelled rRNA (Henderson et al 1972). They can be demonstrated by the argyrophilia of their associated proteins. These proteins are comprised almost entirely of nucleolin (C23) and numatrin (B23), which are associated with the fibrillar centre of the nucleolus (Derenzini et al 1995; Roussel et al 1994).

Silver stained nucleolar organiser regions (AgNORs) demonstrated by the AgNOR technique are seen as tiny black dots which are an integral part of the nucleolus.

Developments in the AgNOR Technique

The AgNOR technique is the silver staining method that demonstrates nucleolar-organised associated proteins. The AgNOR reaction was first described by Goodpasture and Bloom in 1975. Their three step technique was used to stain chromosomes. The chromosomes were treated with a 50% silver nitrate solution followed by ammoniacal silver solution and finally with a developer composed of 3% formalin adjusted to a PH of 5.0 with formic acid. Goodpasture and Bloom (1975) applied this technique to a series of chromosomal spreads. These included chromosomal spreads of animals ranging from *Carollia Perspicillata* (Seba's fruit bat) to *Macaca Mulatta* (the rhesus macaque). Exact concordance was found between

hybridisation sites and the 3-step AgNOR staining. This suggested that the AgNOR technique could be regarded as a nucleolar organiser region labelling method. The original technique of Goodpasture and Bloom was modified by Howell and Black (1980) into a one step sequence. This technique was carried out at 60°C. This method proved to be more reproducible than the original.

Ploton et al (1986) improved the technique of Howell and Black (1980) by lowering the reaction temperature from 60°C to 20°C. They also used the AgNOR technique on cells in smears, chromosomes and semi-thin sections of plastic-embedded human pathological tissues.

Smith et al (1988) investigated the effect of a series of fixatives on the AgNOR technique. Slices of normal fresh tonsil were fixed in a wide range of standard fixatives. Frozen sections were also cut from one slice. The authors concluded that the best results were obtained from Carnoy's fixative, 10% neutral buffered formal saline and fresh frozen sections. The most unsatisfactory results were obtained from mercuric based fixatives such as Helly's and Zenker's fixative. This was probably due to the fact that mercury ions have a great affinity for sulphhydryl groups thus blocking the availability of these to silver ions.

Numerous variables in the actual staining reaction were investigated by McLemore et al (1991). These variables were:

- I. Ultrapure water vs in-house distilled water (D/W)
- II. High-grade silver vs routine histologic grade
- III. Chemically cleaned glassware vs sterile disposable culture tubes
- IV. Percent silver nitrate (AgNO₃): 30% vs 40% vs 50%
- V. High-grade gelatin vs cooking gelatin
- VI. Percent gelatin: 1% vs 2%
- VII. PH of developer: increments of 0.5, from 2.0-5.0
- VIII. PH of 3.0 with formic acid concentration (0.025%)
- IX. Time: 5 min intervals, from 10-60 min

X. Temperature: 4, 25, and 60°C (first under light and then under dark conditions)

XI. Heat: microwave vs convection

Having tested the variables the authors concluded that PH of the staining solution was very important. A PH of 3.0 optimised visualisation by eliminating silver precipitation. Improved contrast between AgNORs and surrounding tissue components was also noted.

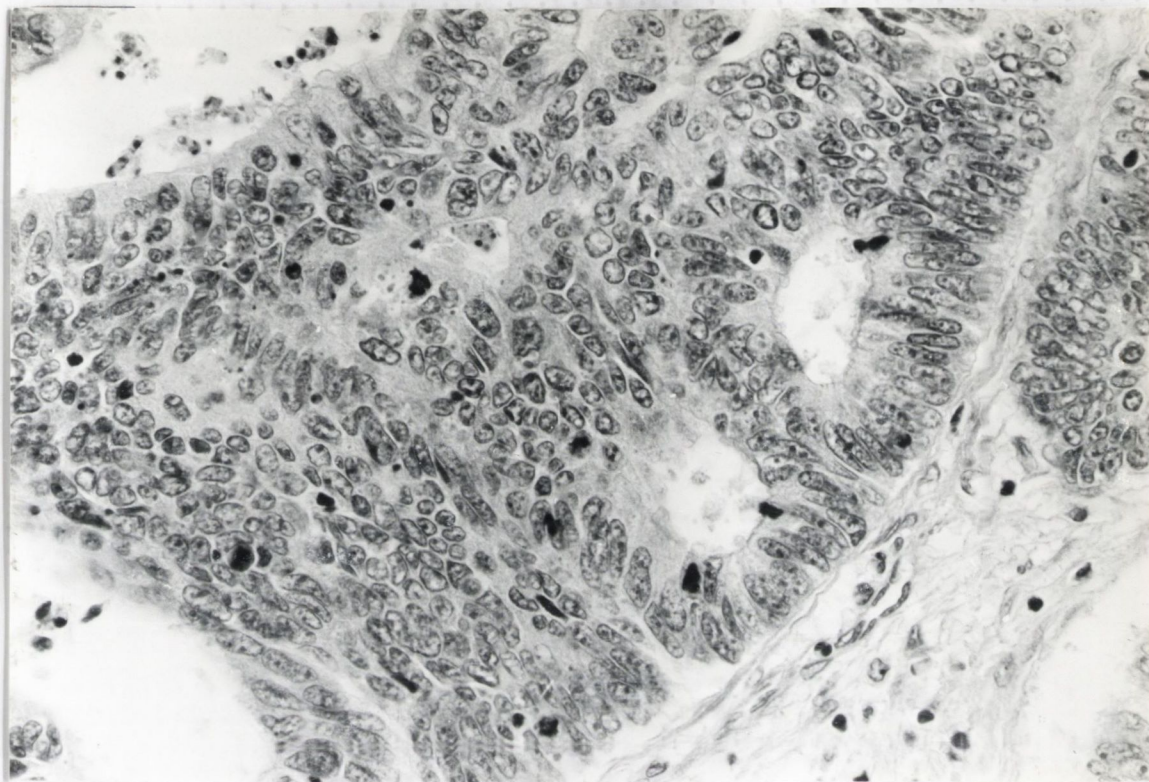
Microwave irradiation was used by Medina et al (1995) to improve silver staining of the AgNOR technique. This study involved the use of animal and plant cells. The authors concluded that both fixation and staining were enhanced after using a microwave. They stated that fixation with ethanol-based reagent, Kryofix, for 3 minutes in a microwave oven resulted in improved structural preservation. The selectivity and contrast of silver-staining was also enhanced. The authors also stated that neither gluteraldehyde fixation nor a treatment of sections with Carnoy's fixative improved AgNOR staining. After an analysis of the effects of the different substances involved in sample preparation Medina concluded that ethanol was an essential factor for fixation for nucleolar staining. In this study semi-thin sections of plastic-embedded tissue were used. Staining was completed by placing a drop of stain on these sections in a microwave oven for one minute. The authors finally concluded that microwave irradiation enhances fixation by controlled heat, whereas the increase in reactivity of the staining solution was a direct effect by the microwaves on the silver ions.

Fixation with the minimum of delay is also important in AgNOR demonstration. In 1998 McDermott et al investigated the significance of a modified nucleolar organiser region silver stain on neoplastic tissue using paraffin and cryostat sections in order to determine the effects of delayed fixation. Samples, 0.8cm in length, of six colonic adenocarcinomas were used for the purpose of this study. For the assessment of the effect of

delayed fixation on AgNOR staining samples of tumour tissue were left in air for the following times: 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours and 48 hours. Samples were also fixed immediately. The tissue blocks were fixed in 10% formol saline for 24 hours. After fixation the tissues were processed, embedded in paraplast and 3 μ m sections prepared. Cryostat sections (5 μ m) of unfixed samples were also cut. These sections were then placed in 10% formol saline for 1 minute. The paraffin and cryostat sections were stained using a modified silver stain. The slides were then scored blind by two of the authors under two headings - intensity of staining and lack of background. The AgNORs were seen as brown/black dots within the nucleolus. The modified silver-staining solution was similar to the solution prepared by Howell and Black with two exceptions. The standard technique did not use a pre-alcohol bath prior to staining or the sodium thiosulphate bath post-staining.

The authors showed that cryostat sections fixed in 10% formol saline prior to staining showed comparable results to paraffin sections that had been subjected to immediate fixation. The cryostat sections did not have any background staining unlike the paraffin sections. Haematoxylin and Eosin sections were prepared of all test samples for identification of the tumour tissue in colorectal carcinoma (figure 1, page 35). The modified staining technique carried out on the cryostat sections is shown in figure 2, page 36. Optimum AgNOR staining results were obtained using cryostat sections that were fixed in 10% formalin for 1 minute prior to staining (figure 3, page 37). Optimum AgNOR results were also seen with paraffin sections of tissue subjected to no more than a 30 minute delay in fixation (figure 4, page 38). There was a significant decline in AgNOR intensity after a 60 minute fixation delay. It was not possible to identify distinct AgNORs following a 24 hour fixation delay (figure 5, page 39). The paper concluded that rapid fixation is essential for AgNOR demonstration in neoplastic tissue.

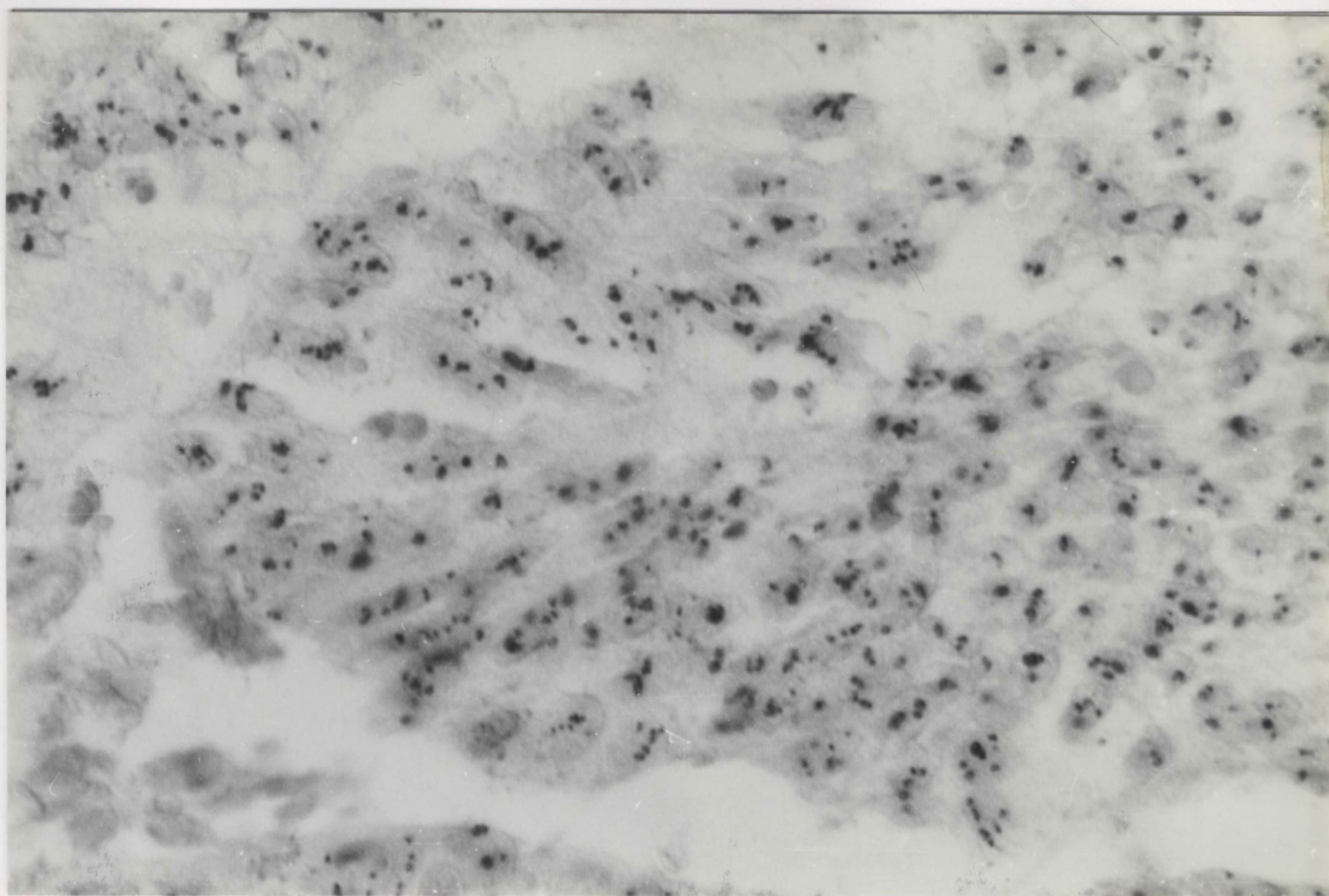
Figure 1: Light micrograph of a paraffin section (haematoxylin and eosin stain) of an adenocarcinoma of colon.



Original magnification x 200.

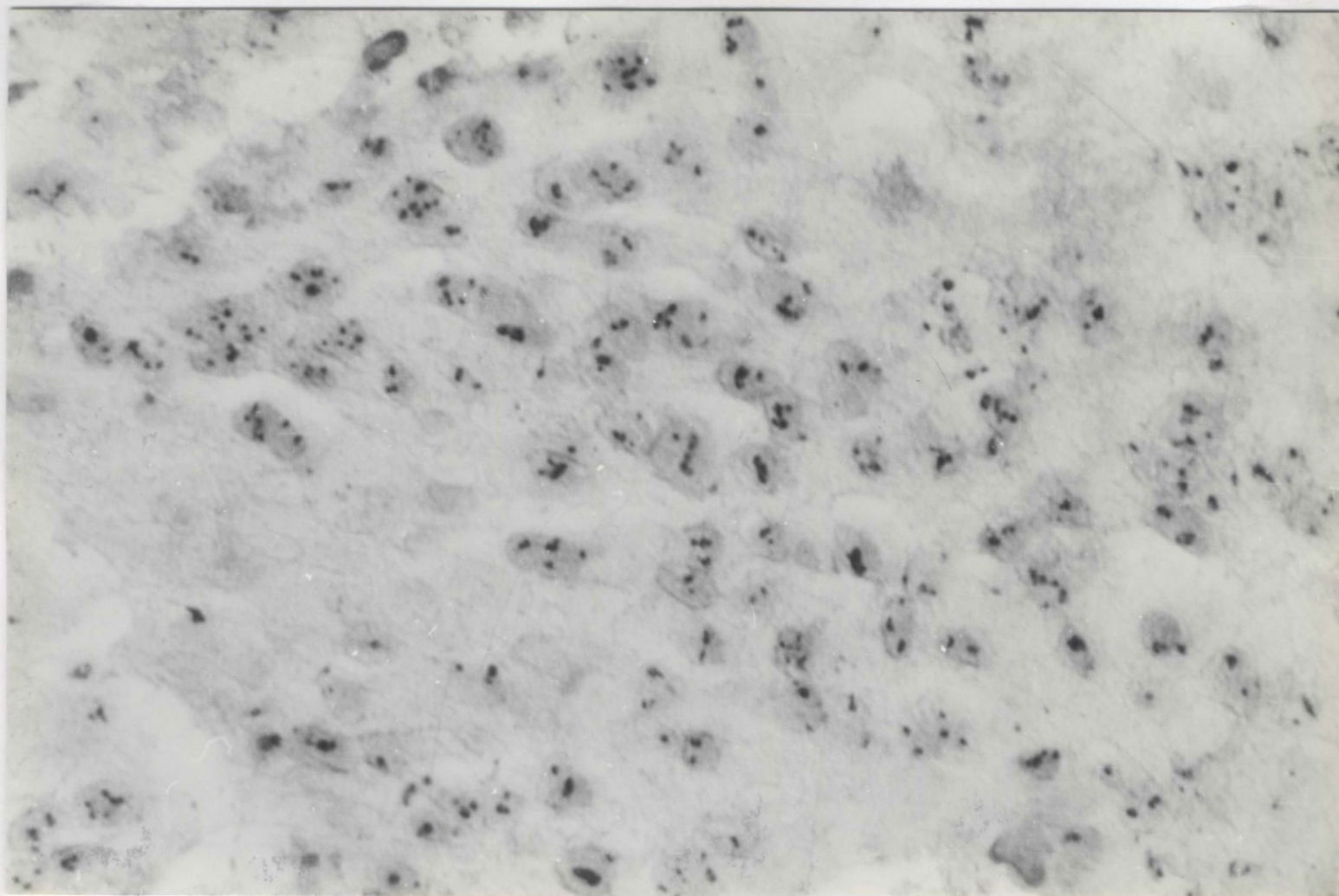
This photo micrograph shows malignant cells arranged in glands, exhibiting a large number of mitosis and moderate pleomorphism.

Figure 2: Light micrograph of a cryostat section (modified AgNOR stain) of an adenocarcinoma of colon.



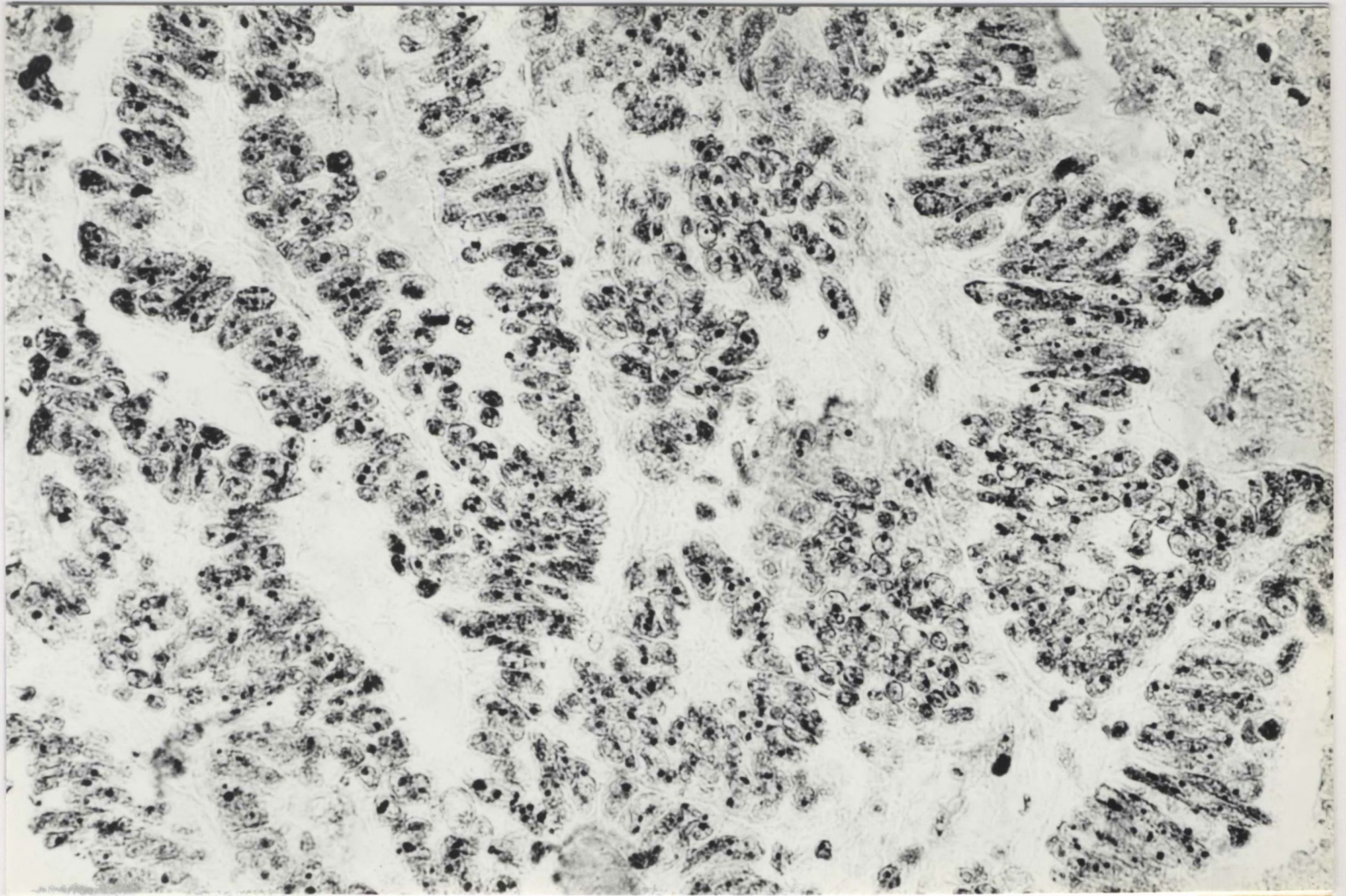
AgNORs are seen as small black dots within the nucleus. Original magnification x 200.

Figure 3: Light micrograph of a cryostat section fixed in 10% formalin for one minute before staining (modified AgNOR stain) of an adenocarcinoma of colon.



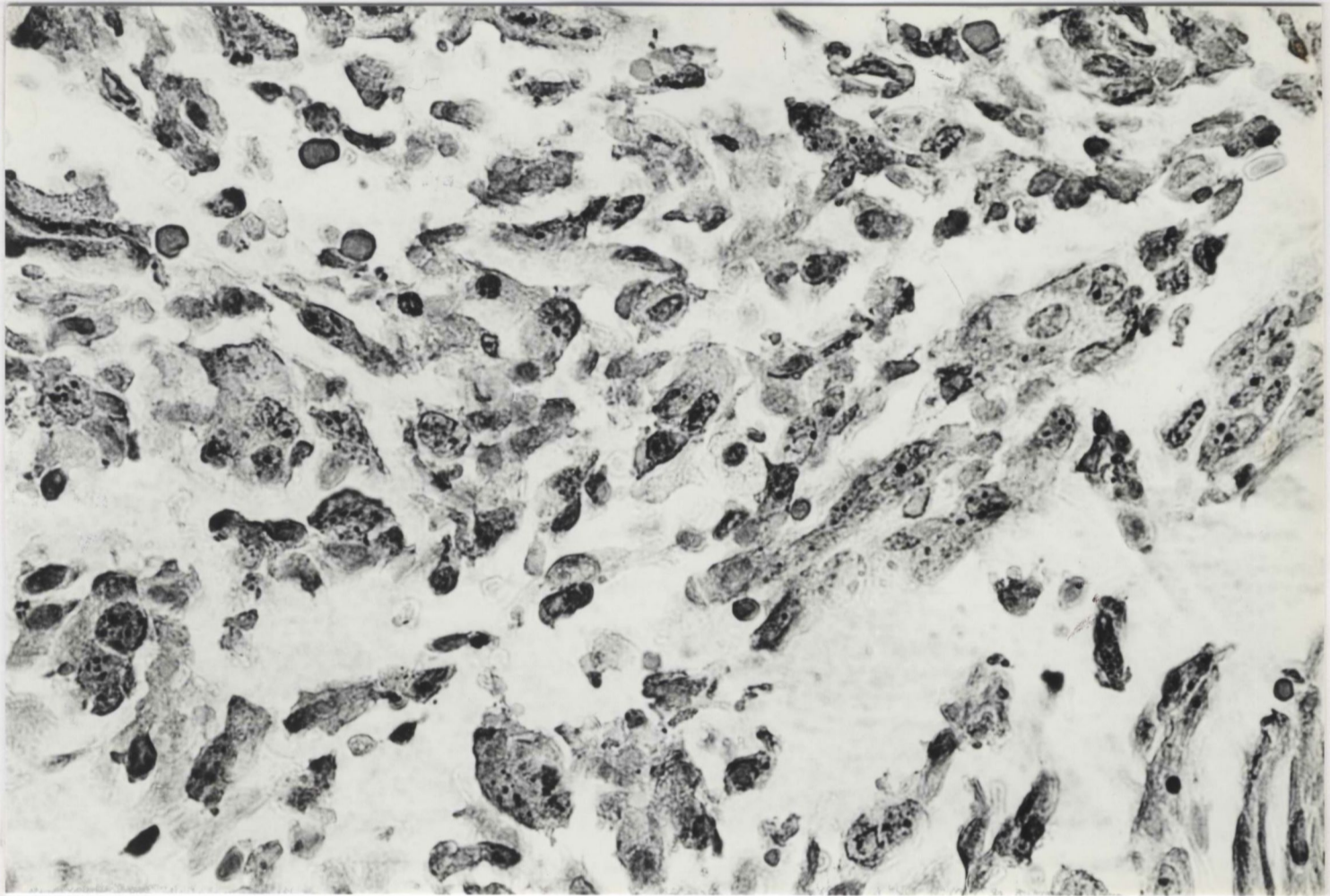
AgNORs are seen as small black dots within the nucleus. Original magnification x 200.

Figure 4: Light micrograph of a paraffin section (modified AgNOR stain) of an adenocarcinoma of colon. Delay in fixation - 30 minutes.



AgNORs are seen as small black dots within the nucleus. Original magnification x 100.

Figure 5: Light micrograph of a paraffin section (modified AgNOR stain) of an adenocarcinoma of colon. Delay in fixation - 24 hours.



AgNORs were difficult to distinguish and impossible to count. This was the result of an excessive delay in fixation. Original magnification x 200.

Immuno-Histochemistry and AgNOR Staining

In 1989 Murray et al introduced a technique which enabled the sequential demonstration of nucleolar organiser regions (NORs) and various antigens in both frozen and paraffin wax-embedded sections. Slices of normal tonsil and lung carcinoma were taken from specimens received fresh from the operating theatre. Cryostat and paraffin sections were first subjected to immuno-staining and then to the AgNOR reaction. This was followed by cryostat and paraffin sections being subjected to the AgNOR reaction and then immuno-staining. This was to assess the importance of sequence in the technique. Good immuno-histochemical and AgNOR staining was obtained when the immuno-histochemical reaction followed the AgNOR sequence. The authors concluded that the main value of the double staining method lies in the ability to ensure that AgNORs are being counted in the desired cell type. They concluded that there was a high correlation between Ki-67 scores and AgNOR numbers.

Morisaki et al (1995) reviewed the immunostaining of the proliferating cell nuclear antigen (PCNA) combined with AgNOR counts in oesophageal cell carcinomas to identify patients with a poor prognosis. PCNA staining provides information about cell kinetics. In this study PCNA was performed on 31 specimens from patients with oesophageal cancer. When an analysis of the results was carried out there was no correlation between PCNA staining and other clinical parameters such as histologic type, lymph node metastases or prognosis. However when an analysis of a combination of PCNA results and AgNOR counts was carried out there was good correlation with patient prognosis. Seven patients with a high PCNA index ($>$ or $=$ 44) and an AgNOR count ($>$ or $=$ 6) had a significantly poorer prognosis than the remaining 22 patients. Six of these seven patients died within two years. This study indicated that a combined PCNA/AgNOR count evaluation may be useful for the identification of patients with a poorer prognosis among those undergoing surgery for

oesophageal squamous cell carcinoma.

The value of PCNA and AgNOR staining in endoscopic biopsies of gastric mucosa was reported on by Irazusta et al (1998). The aim of the study was to examine the usefulness of the quantification of PCNA cells and AgNORs in gastric biopsies for the identification of gastric mucosal proliferative lesions. The test group of 57 paraffin-embedded endoscopic biopsies were classified into four histological groups, i.e.

1. Normal
2. Inflammatory
3. Dysplastic
4. Neoplastic

The PCNA positive cells were identified using immuno-histochemistry. Unfortunately there was no significant differentiation of the four groups using only one of the two parameters. However by using a combination of the results there was a correct classification in 48 out of 57 cases. No correlation between PCNA and AgNOR parameters was found. The authors concluded that the combination of PCNA and AgNOR values may be helpful for differentiating gastric mucosal lesions.

Mechanism of the AgNOR Reaction

In a study of the chemistry of the AgNOR reaction Buys and Osinga (1984) found that AgNORs are coincident with disulphide and sulphhydryl groups of nucleolar organiser region associated proteins (NORAPs). The authors of this research also point out that the silver binding by proteins appears to result from high electron charge density, related to phosphate or carboxyl groups. As it is known that there are many carboxyl groups within the main NORAPs it may be concluded that they are important in the AgNOR binding sequence.

Smith et al (1988) suggested that silver binding in the AgNOR reaction could be sequential. The authors proposed initial binding of silver to carboxyl groups followed by continued nucleation around the disulphide

(-S-S) and sulphhydryl (-S-H) groups. Therefore it appears that both carboxyl and sulphur containing groups are of importance to the AgNOR reaction.

Measurement of AgNOR Area Using Image Analysis

Among the first to measure AgNOR area were Derenzini M et al (1988). This study reported on the distribution of AgNORs as a diagnostic parameter to differentiate between benign and malignant epithelial tumours of the human intestine. The study was performed using electron microscopy and paraffin embedded sections stained for AgNOR proteins. The diameter of the AgNORs was measured using micrographs at a final magnification of x2600. This study concluded that malignant tumours were characterised by a large number of NORs which were small in size and showed a scattered distribution. Nuclei of benign lesions showed a smaller number of AgNORs with a more clustered distribution.

In 1990 Rüschoff et al evaluated nucleolar organiser regions (NORs) by automatic image analysis as a contribution to standardisation. In this study the authors examined the silver staining of nucleolar organiser regions (AgNORs) on 580 tumours from 10 different tissues. The study used an Olympus image analyser. The authors stated that longer staining times produced a higher intensity of AgNOR. They concluded that staining times could be adjusted to the silver-binding characteristics of each block, eg: the most suitable staining time for breast carcinoma was 23-35 minutes. The authors found that an advantage of using an image analyser was that AgNORs were assessed in only one focal plane and section thickness did not significantly influence the result. Inter-observer variability was within 5% error limits.

Variations in the occurrence of AgNORs in non-proliferating and proliferating tissues were reported by Leek et al (1991). In this study the diameter of the AgNORs was measured under the microscope with the aid of a calibrated eyepiece graticule. The authors stated that the number of

AgNOR clusters per nucleus did not correlate well with increased levels of proliferation but AgNOR cluster diameter was consistently related to the proliferative status of cells.

In the same year Trere et al (1991) compared AgNOR area in human tumours with proliferative activity evaluated by bromodeoxyuridine labelling and Ki-67 immunostaining. The area occupied by the silver deposits of 200 cells from each slide was measured using a computer-assisted image analyser (IBAS-KONTRON 2,000). The authors concluded that they demonstrated a significant correlation between the mean area occupied by the AgNOR proteins measured by an image analysis processing system and the proliferative indices evaluated by bromodeoxyuridine labelling and Ki-67 immunostaining. They suggested that AgNOR protein area measurement was a simple, inexpensive, and reliable method for evaluating the proliferative activity in routinely processed samples.

Also in 1991 Derenzini and Trere produced a study on the standardisation of AgNOR measurement by means of an automated image analysis system using lymphocytes as an internal control. The authors used an IBAS-KONTRON 2,000 computer assisted image analysis system for quantitative evaluation of interphase AgNOR areas. Blocks taken from the same lung carcinoma were immersed in four fixatives. These were:

- I. 10% phosphate buffered formalin (PH 7.2)
- II. absolute ethanol
- III. Methcarn solution (methanol-chloroform-acetic acid, 6:3:1)
- IV. Bouin's solution.

After processing the blocks were embedded together in one paraffin block. This meant that after sectioning each section would have a representation from the same tumour treated with 4 different fixatives. Sections were cut from the paraffin blocks and stained using the AgNOR technique. The staining reaction was carried out for 4 separate staining times, i.e. 10, 14, 18 and 25 minutes. The slides were then evaluated using the image analysis

system. Results showed that the intensity of staining varied greatly depending on whether alcohol-containing or formalin-containing fixatives were used. The NORs of cancer cells were more intensely stained in sections fixed in ethanol or Methcarn solution than sections fixed in formalin or Bouin's solution. The mean value of the AgNOR area was also effected by the fixative, i.e. $3.72\mu\text{m}^2$ compared with that measured in the formalin fixed $1.77\mu\text{m}^2$.

The second point the authors made was that the mean AgNOR area from malignant cells increased progressively with staining time. Differences in the value of AgNOR area of non-cancerous cells such as infiltrating lymphocytes were also detected, i.e. the mean AgNOR area of lymphocytes present in tumour tissue lesions was found to vary from 0.26 and $0.35\mu\text{m}^2$ in formalin and in Bouin's fixed tissues to 0.54 and $0.61\mu\text{m}^2$ in ethanol and Methcarn solution-fixed samples. From this paper the authors have shown that:

- I. length of staining time effects AgNOR area
- II. type of fixative, formalin or alcohol, effects AgNOR area
- III. the authors advocate an AgNOR index for malignant tumours. This is simply the mean AgNOR area of the malignant cells divided by the mean AgNOR area of the lymphocyte AgNORs, i.e.

	μm^2 tumour cells	lymphocytes	AgNOR index
Methcarn solution	3.72	0.61	6.10
Formalin	1.77	0.26	6.81

This AgNOR index would then remove the problem of fixative variations or staining times. This would enable labs to compare similar results rather than that of different fixatives and staining times.

Three years later in 1994 Gonzales et al demonstrated that AgNOR

area measurements differentiated benign and malignant melanocytes more accurately than simple counting. The authors studied AgNOR area in 32 melanocytic skin lesions using an Olympus CUE 2 with morphometry software on an IBM 286 AT computer connected to a Zeiss microscope. The AgNOR area/nuclear area ratio was found to be a discriminating factor between melanoma and all other benign groups studied. They found this ratio discriminated benign from malignant melanocytic lesions better than AgNOR counts.

In 1995 Nakayama et al reported on AgNOR area per nucleus as a prognostic factor in breast cancer. The authors stated that at the time of their study AgNOR evaluation as a predictor of prognosis in breast cancer was controversial. In order to evaluate the technique several AgNOR variables were reviewed in 131 patients with breast cancer. They measured the mean area of AgNOR 'dots', the mean AgNOR area per nucleus (MA), and the mean AgNOR number per nucleus (MN). These evaluations were carried out using automated image analysis. Overall survival was better in patients with lower MA than those with higher MA. They concluded that the mean area of AgNORs was an important prognostic factor.

Also in 1995 Trere et al demonstrated that morphometric analysis was more highly reproducible than the counting method for AgNOR quantification. They studied AgNOR quantity both by measuring the area of the silver-stained structures using image analysis and counting AgNOR numbers directly from the microscope. Two observers examined each slide. They found that measuring AgNOR area using image analysis was more reliable and reproducible than the AgNOR counting method.

A comparison of AgNOR protein quantity of cervical smears and histological sections in cervical intraepithelial neoplasia was made by Pelusi in 1997. The authors used image analysis to compare AgNOR area in normal cervix, CIN 1, CIN 2 and CIN 3. No significant difference was demonstrated in the mean AgNOR protein area values between normal

cervix, CIN 1 and CIN 2 while AgNOR protein scores of CIN 3 were significantly greater than those of normal cervix. The authors concluded that AgNOR protein mean area may be useful for the cytological diagnosis of cervical lesions.

Nucleolar Organiser Region Pattern Assessment

A scoring system was devised in 1993 by Meehan et al to evaluate silver-stained nucleolar organiser regions in cytological preparations of benign and malignant breast lesions. In this study it was acknowledged that previous projects had been applied to benign and malignant lesions in a wide range of tissues and it was generally accepted that there are significantly greater numbers of AgNORs in malignant rather than in benign lesions. The study went on to suggest a scoring system that could be used to test the diagnostic value of AgNOR pattern assessment in conjunction with counting, in breast cytological preparations.

Random smear preparations were selected from 200 fresh breast specimens that had been sent for frozen sections over a two year period. The cut surface of the breast tissue sent for frozen section was scraped with a scalpel. A smear was then made on a glass slide and then fixed in absolute alcohol for one minute. The smears were then stained using the AgNOR technique. Nuclei were counter-stained lightly in 0.5% methyl green. The AgNORs were seen as black dots within the nucleus. Counting was carried out by two observers. Using a scoring system the observers gave points under the following parameters.

- A) estimated number of AgNORs per cell
- B) variation in satellite size
- C) variation in satellite shape
- D) variation in cluster size
- E) variation in cluster shape

Each case scored a minimum of 5 (1 point for each parameter) or a maximum of 15 (3 points for each parameter). The authors noted that with

practice speed and inter-observer reproducibility improved. The study showed that an AgNOR count per nucleus of 8 or more designated malignancy with a diagnostic accuracy of 85%. A pattern assessment score of 10 or more were malignant with a diagnostic accuracy of 85%. Using a combination of both techniques 31 out of 36 malignant cases and 59 out of 62 benign cases were correctly diagnosed with an accuracy of 90%. The authors concluded that the diagnostic value of AgNORs alone was probably not useful in differentiating benign from malignant breast lesions. However they reported that the technique may be of use in giving additional diagnostic information to pathologists.

Examination of AgNORs Using the Scanning Electron Microscope (back-scattered mode)

In a study by Thiebaut et al (1984) the authors used the scanning electron microscope to study cultured cells stained with the AgNOR technique. Cells were grown on coverslips, stained with silver, dehydrated with ethanol and critical point dried using liquid nitrogen. The coverslips were then carbon-coated and analysed with a Phillips SEM 500 operated at 50KV for the back-scattered electron image. The authors concluded that the AgNOR technique in combination with the SEM (back-scattered mode) could provide some additional information about surface structure imaging.

Examination of the Three Dimensional Display of AgNORs by Confocal Laser Scanning Microscope

Recently a new method of fluorescent staining of nucleolar organiser regions (FiNORs) has been described (Imamura et al 1993). Aluminium ammonium sulphate was used as the cationic metal ion for the binding of NOR-associated proteins. Fluorescent morin was then bound to aluminium by chelating. The standard AgNOR technique was then carried out. Both images of FiNORs and AgNORs coincided with each other.

Histopathological Applications of Silver Stained Nucleolar Organiser Regions on Tumour Tissues

Non-Hodgkin's Lymphomas

The grading of non-Hodgkin's lymphomas is an important but complex procedure. Electron microscopy, enzyme histochemistry, immuno-histochemistry, morphometry and DNA flow cytometry have all been used in their assessment. Crocker and Nar (1987) showed that the number of AgNORs was significantly greater in high grade lymphomas (4.4-6.8 AgNORs per nucleus) as opposed to low grade lymphomas (1.0-1.5 AgNORs per nucleus). A similar study (Boldy et al 1990) confirmed these results using cell imprints of lymphoid tissue. The advantages of cell imprints is two-fold:

1. The examination of whole cells gives a more realistic count with regard to the total numbers of AgNORs per nucleus (Rüschhoff et al 1990).
2. A greater distinction between low and high grade non-Hodgkin's lymphomas can be made.

The relationship between AgNOR and cell proliferation counts was studied by Hall et al (1988) and Crocker et al (1988) using the monoclonal antibody Ki-67. Hall et al (1988) labelled 80 non-Hodgkin's lymphoma frozen sections with Ki-67 and carried out AgNOR stains on the subsequent paraffin sections. The percentage of cells reacting with Ki-67 and the mean numbers of AgNORs per nucleus were compared. This comparison showed a good correlation of results. The advantages the AgNOR technique has over the immuno-histochemical technique is that it does not require frozen tissue and it can be carried out on routine paraffin blocks.

A quantitative analysis of AgNORs to assess their potential in differentiating the grade of non-Hodgkin's lymphomas was carried out by Yekeler et al (1993). There were highly significant differences in AgNOR counts among the various grades. They concluded that the method was useful in diagnostic histopathology. Also in 1993 Jakic-Razumovic et al

investigated the relationship between the numbers of nucleolar organiser regions and survival in patients with non-Hodgkins lymphomas. The AgNORs were counted in 150 nuclei of each of the 61 specimens examined. The authors concluded that the numbers of AgNORs were highly predictive for survival, remission and the length of remission in patients with non-Hodgkins lymphoma. Two years later the same author, Jakic-Razumovic et al (1995), studied the AgNOR predictive value of prognosis in non-Hodgkins lymphoma according to the Kiel classification. Specimens from 58 patients were studied. It was shown that the mean number of AgNORs differed between low and high grade non-Hodgkins lymphoma along with survival, The value of 2.9 NORs per nuclei was the cut off point for separating the whole group into two sub-groups, which differed the most when related to survival of patients with non-Hodgkins lymphoma.

In 1995 Hendricks et al compared computer assisted and visual methods of assessing ceilular proliferation in tissue sections from non-Hodgkins lymphomas. The study group consisted of 10 indolent and 10 aggressive tumours. The results demonstrated that there was a significantly higher number of AgNORs in aggressive tumours compared to indolent non-Hodgkins lymphomas. However the correlation between these counting methods (computer assisted and visual) was not significant.

Skin Lesions

Using the AgNOR silver-staining technique, Crocker et al (1987) established that the AgNOR numbers were similar in malignant lesions while there was a significantly lower number in benign naevi. One year later Howatt et al (1989) established that it was not possible to differentiate between types of thick cutaneous carcinoma. He also reported technical and non-reproducibility problems. Another study (Mackie et al 1989) examined AgNOR counts in benign naevi and malignant melanoma. The difference in AgNOR counts between all types of naevi and all types of melanoma was statistically significant but there was no difference between

benign naevi and dysplastic naevi. The author recommended the AgNOR technique to differentiate between true melanoma and borderline melanocytic naevi.

A re-evaluation of silver-stained nucleolar organiser regions (AgNORs) in problematic cutaneous melanocytic lesions was carried out by Evans et al (1992) using quantitation and pattern analysis. The authors suggested that inconsistencies in AgNOR results in skin lesions have come about because of differences in fixation, staining time and counting techniques. This work optimised counting of AgNORs using intra and extra nucleolar parameters. This made it possible to discriminate dysplastic naevi from melanoma and also distinguish between Spitz naevi and pigmented spindle cell naevi from melanoma.

The theory that AgNOR area measurements differentiate benign and malignant melanocytic lesions more accurately than simple counting was proposed by Gonzales et al (1994). The authors found that AgNOR counts had been found to give significant differences between benign and malignant conditions. Unfortunately they also found a considerable overlap. They concluded that the ratio of AgNOR area/nuclear area discriminates benign from malignant melanocytic lesions better than AgNOR counts. Also in 1994, Ronan et al investigated the prognostic significance of AgNORs in melanomas. In the study the authors counted AgNORs in 26 skin paraffin sections from 26 patients with primary cutaneous melanoma. Fourteen of the patients were still alive without the disease 5 years after diagnosis. Twelve of the patients were dead in less than 5 years. AgNORs were scored in 30 nuclei per tumour. In the study the AgNOR counts did not prove to be of prognostic value in malignant melanoma.

Using image analysis nuclear morphometry, Kossard et al (1995), studied AgNORs in small cell (naevoid) melanoma. The authors reported that AgNOR area was not useful in distinguishing between superficial

spreading melanomas and dermal naevi, however, there was a significant difference in AgNOR numbers between the two groups.

Two years later Naik et al (1997) reported on a AgNOR study in melanocytic skin tumours. Thirty five cases of melanocytic skin tumours were reviewed. In 23 cases of benign melanocytic naevi the mean AgNOR number/nucleus was between 1.06 and 3.43 with a mean of 2.05. In the 12 cases of malignant melanomas the values were between 4.26 and 10.66 with a mean of 7.43. Because statistically different AgNOR counts were found between the two groups the authors suggested the technique may serve as a useful adjunct to routine histopathology.

Breast Lesions

AgNOR counts on both benign and malignant lesions and normal tissue were carried out by Smith and Crocker (1988). The AgNOR sites were counted in three different ways:

1. The total number of AgNORs were counted from each nucleus.
2. "Clumps" of AgNORs were examined.
3. The numbers of AgNORs per clump were counted.

Only method one (the total AgNOR count) was useful in distinguishing between the benign and malignant groups. In a subsequent analysis (Giri et al 1989) which assessed AgNOR clusters as single units found overlap between benign and malignant cases.

In 1992 Herir et al investigated the use of the AgNOR technique as a prognostic indicator in breast carcinoma. AgNOR counts were carried out in 48 cases of breast carcinoma. Archival specimens were used. The AgNORs were counted in 100 cells by one observer under a 100x objective lens. The range of AgNORs varied from 1.1 -10.2 with a median value of 3.5 per cell. Patients were then classified into two groups:

Group A: mean AgNOR count > 3.85

Group B: mean AgNOR count < 3.85 or = 3.85

A significant difference in a 5 year survival rate was found between

Groups A and B. The 5 year survival in Group A was 21% and the five year survival in Group B was 85%. The authors concluded that nucleolar organiser regions may be useful as a prognostic indicator in breast carcinoma patients.

A study designed to evaluate the use of NORs as a prognostic factor in infiltrating ductal carcinoma of the breast was carried out by Canepa et al (1993). The authors found a significant correlation between AgNOR score and the outcome of the disease. By means of discriminant analysis a threshold value of AgNOR score was identified (9.5). This distinguished two risk groups among the cases under examination. Those cases below the threshold value showed a favourable prognosis. The cases with an AgNOR score greater than 9.5 had an unfavourable outcome. The authors suggest that AgNOR score may be used as a prognostic parameter in breast carcinoma.

In 1993 Toikkanen and Joensuu suggested that AgNOR counting did not appear to be a useful prognostic variable in breast cancer. The authors carried out AgNOR counts on 370 diagnosed cases of breast carcinoma. Sections of 4 μ m were stained by the method of Smith and Crocker with minor modifications. Counting of the AgNORs was carried out without any knowledge of clinical data with an oil immersion objective at a total magnification of 630x. The authors found that the AgNOR count had no predictive value in relation to a 5 year or 8 year survival. They also stated that the AgNOR counts had no relationship with histological grade or type, mitotic count, tumour size or axillary node status. They concluded that routine AgNOR counts had little to add to the spectrum of prognostic features in breast carcinoma.

Research investigating the diagnostic relevance of AgNORs in discriminating between benign and malignant breast lesions was carried out by Agarwal et al (1995). The paper also attempted to ascertain the inter-observer variability in the enumeration of AgNOR counts. The work

demonstrated a correlation between AgNOR counts and the size of tumour axillary lymph node status and age of the patient. The results showed close agreement between the two observers. The authors concluded that AgNOR counts are of little diagnostic importance since a broad zone of overlap exists between benign and malignant breast lesions despite significantly higher mean counts in malignant lesions.

Two years later Kumar et al (1997) investigated the value and correlation of AgNORs as clinical prognostic factors in breast carcinoma. In this study 46 patients with primary carcinoma were correlated with clinical parameters of breast cancer. Specimens from 10 patients with benign breast tumours were used as controls. Sections from the benign and malignant breast tumours were stained using the AgNOR technique and the number of AgNORs from within the nuclei of 100 tumour cells were calculated in each specimen. The results were expressed as +/- SD. The results from the study showed a statistically significant increase in correlation with the increase in the size of tumour, stage of the cancer, number of metastatic lymph nodes and tumour recurrence at various sites. The AgNOR count in breast carcinoma was 6.61 ± 1.75 and 1.88 ± 0.19 in benign tumours. The authors stated that breast tumours with a high AgNOR count, even at the initial stage, have a poor prognosis and require aggressive treatment. It was also suggested that patients with a benign tumour and more than three AgNORs per nucleus need careful surveillance.

Also in 1997 Bellotti et al carried out a morphometric determination of AgNORs in breast carcinoma and correlated their results with ploidy using flow cytometry. They also correlated a morphometric analysis of AgNORs with proliferating cell nuclear antigen (PCNA). Twenty five cases of infiltrating ductal carcinoma of the breast were studied. Several parameters were quantified using image analysis. These parameters included nuclear area, mean value of the number of AgNORs, area of

AgNORs and area of AgNOR per nucleus. Comparison of AgNORs with the size of tumour and lymph node status was also made. The slides were measured using an Olympus BH-2 analysis system using a 100x objective. For each case 100 cells were examined. The results showed good correlation between both the mean number of AgNORs and the mean AgNOR area and ploidy. There was also correlation between the coefficient of variation of AgNOR area per nucleus and proliferation cell nuclear antigen, PCNA. The authors concluded that the AgNOR technique will eventually be used as a prognostic indicator in breast cancer.

Breast Aspirates

The diagnostic value of silver nucleolar organiser region assessment in breast cytology was studied by Meehan et al (1993). The authors applied the silver nucleolar region technique to cytological preparations obtained from surgical specimens to evaluate the usefulness of the method in distinguishing between benign and malignant breast lesions. As well as counting the AgNORs a subjective scoring technique was used to assess AgNOR size, shape and clustering. The benign lesions had a mean count of 4.44 AgNORs/nucleus and a subjective score of 7. The malignant lesions had a mean count of 9.52 and a score of 13. The diagnostic accuracy of combined counting and scoring was 90%. Although the accuracy of this evaluation is high the authors felt from a diagnostic point of view AgNORs alone are probably not useful in differentiating between benign and malignant breast conditions. This is because of an overlap in counts between the clear-cut benign and malignant cases studied. However they felt that the AgNOR counting and scoring gave additional diagnostic information in cases that presented difficulty using routine stains. They also felt that their technique may be of use in fine needle aspiration.

The limitations of their combined scoring and counting technique was evident in malignant lesions with low mean AgNOR counts (range 2-8 AgNORs/cell) and low scores (5-10). The study concluded with the

observation that their technique may tell more about the proliferative behaviour of a tumour rather than benignity versus malignancy.

An analysis of AgNOR counts in a range of breast lesions was carried out by Karmakaer et al (1995). The counts were carried out on fine needle aspiration smears (FNA). Seventy-two breast lesions were examined. These included ductal carcinomas, fibrocystic change, fibroadenoma, proliferative lesions and miscellaneous lesions. The ductal carcinomas showed higher AgNOR counts (mean 16.63 7.09) compared with non-malignant lesions (6.39 1.96). The differences were statistically significant. A cut off AgNOR score of 11 could reliably discriminate between benign and malignant lesions. The research also showed that in ductal carcinomas the AgNOR score showed a tendency to increase with higher grades of malignancy. The authors also concluded that AgNOR counting, although tedious, is inexpensive and provides useful information regarding cell proliferation and can supplement information obtained by more sophisticated techniques.

An evaluation of the usefulness of the AgNOR technique was carried out by Dasgupta et al (1997) who reviewed AgNORs in 30 fine needle aspiration smears (FNAC) and 45 tissue sections from benign and malignant breast lesions. In the fibrocystic disease cases the mean AgNOR count was 1.6 (FNAC group 0.75, tissue section 1.61). In the fibroadenomas it was 1.61 (FNAC group 1.63, tissue section 1.59). In the carcinomas the mean count was 12.10 (FNAC 12.08, tissue section 12.10). The difference in AgNOR count between fibrocystic disease and fibroadenoma was not significant, however, there was a significant difference between benign breast lesion and carcinoma. No significant difference was found between FNAC and tissue sections in either the benign or malignant groups. The authors concluded that the technique could be used as an additional criterion to differentiate the benign and malignant lesions of breast.

Cervical Smears

In the same year Calore et al (1997) destained Papanicolaou stained cervical smears and applied the AgNOR technique to them in order to evaluate it's value as a diagnostic aid. The smears were made from benign and preneoplastic lesions. AgNOR counts were performed on six normal, six inflammatory, six squamous inetaplasia, six CIN 1, six CIN 2, and five CIN 3 smears. Statistically significant differences were found in each of the groups examined. The authors reported that the AgNOR technique could be useful to evaluate cervical smears of doubtful interpretation using previous demarcation of the abnormal fields/cells.

Gastrointestinal Neoplasia

Derenzini et al (1988) examined the distribution of the interphasic nucleolar organiser regions (NORs) in hyperplastic polyps, adenomatous polyps and colonic carcinomas. The investigation was carried out using silver stained sections in combination with electron and light microscopy. Malignant tumour cells were characterised by a large number of small NORs showing a scattered distribution. Nuclei of both types of polyp had only a small number of large-sized NORs with a distribution intermediate between that of benign and malignant. While statistical differences were observed, the large overlap between the categories indicates preclusion of the use of AgNOR counting as an accurate method of distinguishing benign lesions from malignancy. Moran et al (1989) investigated the prognostic value of nucleolar organiser regions in advanced colorectal cancer. This study indicated that nucleolar organiser region counts have considerable potential as a prognostic indicator in advanced colorectal cancer.

Four years later Joyce et al (1992) investigated the 5 year prognostic value of AgNORs in colorectal cancer. Each patient was categorised using the classical method, i.e. Dukes staging and a mean AgNOR score was determined for their tumour. The tumours were well differentiated, moderately differentiated and poorly differentiated non-survivors. The

authors concluded that the AgNOR score was a significant predictor of survival in patients with colorectal tumours. In 1994 Hennigan et al reviewed the prognostic value of nucleolar organiser regions in colorectal neoplasia. The authors counted AgNORs in normal colonic mucosa, adenomas and carcinomas. The study also reported on the relationship between AgNOR numbers and adenoma diameter, carcinoma stage, patient survival and tumour recurrence. The conclusion of this study stated that there were significant higher AgNOR counts in carcinoma (median 3.64) and adenoma (median 2.3) compared to non-neoplastic mucosa (median 1.96). However NOR frequency was not significantly related to adenoma diameter, carcinoma node status, patient survival or tumour recurrence and was therefore of no value in prediction of adenoma or carcinoma behaviour. Ofner et al (1995) demonstrated that higher numbers (>4) of scattered, AgNORs (in those not occurring as nucleolar clumps) were significantly associated with poor survival in colonic cancer. Also in 1995 Adachi et al studied nuclear DNA content and nucleolar organiser regions in colorectal cancer. Their paper disagrees with the work of Ofner et al in that they found no correlation between AgNOR counts and survival of patients. It is possible that because they only counted "distinct dots" and did not use a wet autoclave for pre-treatment they would not be examining the same parameters as that of Ofner. One year later in 1996 Ikeguchi et al reported on proliferative activity of cancer cells at the invasive margin of a tumour and as an indicator of prognosis of patients with gastric cancer with serosal invasion using the AgNOR technique. Five sites along the invasive margin of the tumour were assessed in 65 patients with histologically proven gastric adenocarcinoma that had invaded the serosa. The mean number of AgNORs at the invasive margin of the tumour was 4.6. Patients with a margin of tumour AgNOR scores of 4.6 or above had a 5 year survival rate of 22.7%. Patients with a low AgNOR score had a 5 year survival rate of 55.4%. The authors concluded that the AgNOR score for

malignant cells at the invasive margin appeared to be a good predictor of the prognosis of patients with advanced gastric carcinoma with serosal invasion.

Histopathological Applications of Silver Stained Nucleolar Organiser Regions on Cells and Tissues which have been Subjected to Radiation Treatment

A morphometric study of AgNORs was carried out on an experimental model of irradiated squamous epithelium (Wistar rat sole skin), 4, 8 and 14 hours and 1, 2, 5 and 7 days post-irradiation with 50Gy of X-rays (Schwint et al 1993). A statistically significant rise in AgNOR volume of up to 238% and reduction in AgNOR number/nucleus of up to 40% was detected as a function of post-irradiation time. A statistically significant 46% increase in the AgNOR volume was detected as early as 8 hours post irradiation, when no histological changes were observed in routine preparations. In this investigation the decrease in AgNOR numbers/nucleus reflected the inhibition of transcription from ribosomal DNA (rDNA) to ribosomal RNA (rRNA).

One year later Miller et al (1994) determined the prognostic significance of AgNORs in endometrial malignant tumours. Decreased AgNOR numbers following radiation therapy was demonstrated, presumably because transcription in the tumour cells was inhibited.

In 1996 Babu et al carried out an investigation to establish the prognostic significance of argyrophilic nucleolar organiser regions in oesophageal carcinoma. Fifty patients were studied. All had been treated with pre-operative radiotherapy. An analysis was carried out for the presence of change in AgNOR numbers before and after radiotherapy. It was found that on average AgNOR numbers per nucleus were lower in patients after radiotherapy (2.89 +/-1.04) than before radiotherapy (3.17 +/- 9.69). This suggested radiotherapy caused a reduction in AgNOR numbers. It was noted that patients with an AgNOR count of < or = 3.0 had

a mean survival of 30.39 +/- 3.29 months and those with a mean count of > 3.0 per nucleus had a mean survival of 27.8 +/- months. Two conclusions were made:

1. AgNOR number may be helpful in determination of prognosis in small patients with oesophageal carcinoma. et al 1992). The purpose of the cur
2. Preoperative radiotherapy seems to decrease AgNOR count with radiat improved survival.

testine. This additional information may then be used to provide a more complete understanding of total radiation damage of tissue. A scanning electron microscope paper was completed by Thiebaut et al (1984), on AgNORs in cultured neoplastic cells. As a similar SEM preparation technique was utilised in this study, it seemed appropriate to include neoplastic tissue as an interesting addendum to the current research.

Objectives

1. To measure using an image analysis system, the mean AgNOR area/nucleus within the various compartments of control small intestine samples (jejunum).
2. To examine post-irradiation variations in the mean AgNOR area/nucleus within mouse small intestinal tissue samples using 5Gy, 10Gy, 15Gy and 20Gy doses of gamma radiation at time points ranging from 30 minutes to 72 hours.
3. To investigate if a relationship exists between the mean AgNOR area/nucleus post-irradiation values obtained from this research and mean AgNOR number/nucleus results from a previous study.
4. To observe and compare the structure of silver-stained nucleolar organiser region associated proteins in normal, irradiated and tumour samples using the scanning electron microscope (standard emission & back-scattered mode).

6. AIMS and OBJECTIVES

Rationale

It has been shown that a complex chain of events takes place in the small intestine following irradiation (Carr et al 1992). The purpose of the current research was to provide further information on the effect of radiation on the small intestine. This additional information may then be used to provide a more complete understanding of total radiation damage of tissue. A scanning electron microscope paper was completed by Thiebaut et al (1984), on AgNORs in cultured neoplastic cells. As a similar SEM preparation technique was utilised in this study, it seemed appropriate to include neoplastic tissue as an interesting addendum to the current research.

Objectives

1. To measure using an image analysis system, the mean **AgNOR area/nucleus** within the various compartments of **control** small intestine samples (jejunum).
2. To examine **post-irradiation** variations in the mean **AgNOR area/nucleus** within mouse small intestinal tissue samples using 5Gy, 10Gy, 15Gy and 20Gy doses of gamma radiation at time points ranging from 30 minutes to 72 hours.
3. To investigate if a **relationship** exists between the mean **AgNOR area/nucleus** post-irradiation values obtained from this research and mean **AgNOR number/nucleus** results from a previous study.
4. To observe and compare the **structure** of **silver-stained nucleolar organiser region** associated proteins in **normal, irradiated** and **tumour** samples using the **scanning electron microscope** (standard emission & back-scattered mode).

Part 2.

MATERIALS and METHODS

1. SPECIMENS

Six week old male BSVS mice were used in this research. The animal studies were carried out by a licensed investigator in accordance with the British Council's Guidelines on the use of living animals in Scientific Research, 2nd Edition. The test animals were taken to the Cobalt -60 theratron teletherapy device situated at a different site for irradiation. The mice were then returned to their home environment and killed by cervical dislocation. Groups of six mice were sacrificed at the following time points after irradiation:- 30 minutes, 6 hours, 12 hours, 24 hours and 72 hours. In addition, six non-irradiated controls and one pre-stressed non-irradiated control (sham) per dose were also studied. Sham animals were also taken to the Cobalt-60 Theratron Teletherapy device situated at the radiation centre. However, they were not irradiated. The purpose of the shams was to show that AgNORs in these animals were not affected by the stress of travelling or handling. Control animals were subjected to minimal stress and not moved from their home environment.

2. IRRADIATION TECHNIQUE

The mice were placed in a ventilated plastic box and were whole body exposed to 5, 10, 15, and 20 Gy gamma-radiation from a Cobalt-60 Theratron Teletherapy device, working at a dose rate in the range 113-145 cGy/minute. Since the radioactive source decays continuously, dose rates are calibrated monthly. The gamma rays produced are equivalent to X-rays at 2.5-3.0 MeV.

3. FIXATION

Normal and irradiated intestinal samples of jejunum were fixed for 24 hours in 10% formol saline using an inflation method described by Carr and Toner (1972). Briefly, samples were flushed, inflated gently with the fixative, the ends tied off and the segments immersed in the fixative.

Cross sections of mid-jejunum was used for study. The jejunum was selected because part of the current research was to compare results with previous work that utilised this area of the small intestine.

4. TISSUE PROCESSING

The tissues were processed on a Shandon Hypercenter, embedded in paraplast and 4 μ m sections were cut on an Anglia Scientific Rotary Microtome. The sections were then dried for three hours in a 60°C oven and stained by a modified AgNOR technique (Howell and Black 1980).

5. MODIFIED AgNOR STAINING TECHNIQUE

Paraffin sections were hydrated, placed in three changes of deionised water, then immersed in absolute alcohol for thirty minutes. The sections were washed in deionised water and placed in the staining solution in the dark for 35 minutes at 20°C. The staining solution consisted of a filtered 50% silver nitrate solution (2 parts) and 1% fresh formic acid (90% solution) in 2% gelatin (1 part). A PH of 3.5 was found to be optimal for the reaction. The sections were then thoroughly washed in deionised water and placed in 5% sodium thiosulphate (5 minutes), washed again and finally dehydrated, cleared and mounted. The AgNORs are seen as brown-black dots within the nucleolus. The staining solution was similar to that prepared by Howell and Black (1980) with the exception of the pre-alcohol bath prior to staining and the sodium thiosulphate bath post-staining.

6. MEASUREMENT of AgNOR AREA

The area occupied by the silver deposits of 100 cells for each slide was measured using a specific computer-assisted image analysis system (IBAS Kontron 2000). A field was selected by scanning the slide on the microscope with a x40 oil immersion objective lens. The selected image was then captured into digital memory and visualised on a colour monitor.

On the monitor the silver-stained NORs appeared as black structures of different shapes and sizes, distributed throughout the nucleus, contrasting with the lighter background. By moving the mouse on the digitiser tablet, the grey threshold was defined. This threshold value was totally dependent on the operator. The mouse was moved on the digitiser tablet (mouse pad) until the area covered by AgNORs was highlighted on a colour monitor. When the operator was satisfied that all of the AgNORs were highlighted on the monitor the mouse was pressed and image capture and segmentation took place. The AgNOR area was then automatically measured.

Unfortunately in Histology Image Analysis is disadvantaged by the complexity of histological images which causes difficulties in the automated detection of the objects to be measured, ie: if there is any black silver precipitate present it is difficult not to include this in the image analysis of AgNOR area. The setting of the threshold limit differs slightly from one observer to another, depending on powers of observation, quality of microscope and available software. However, advances in image analysis systems will in future minimise human error in the setting of threshold levels.

7. COUNTING of AgNORs - (from a previous study, J.G. McDermott MPhil, Queens University, Belfast, 1997)

The AgNOR numbers/nucleus were counted from within the nucleus of one hundred cells/region of the small intestine. These regions were the upper and lower villus epithelium, the lamina propria, the crypt epithelium and the muscularis externa. The mean number of AgNOR numbers/nucleus in each of the regions was calculated separately. The counting was carried out blind on coded sections. The type of specimen, post-irradiation time points, doses, irradiation, fixation, tissue processing and staining utilised in the previous research were the same as those in the current study.

8. STATISTICAL ANALYSIS

Data was entered into a one way analysis of variance (ANOVA) and multiple comparisons between means assessed using the T method. For graphical representation, upper and lower 95% comparison intervals were calculated. Means are significantly at p 0.05 if, and only if, intervals do not overlap.

9. EXAMINATION of the INTERNAL STRUCTURE of the AgNOR USING the SCANNING ELECTRON MICROSCOPE (standard emission & back-scattered mode) - specimens

i. Normal samples

Six week old BSVS mice were used in this study. The mice were killed by cervical dislocation. Samples from the middle portion of jejunum were used from six mice and treated in the same manor as in the previous investigation.

ii. Irradiated samples

Jejunum samples from BSVS mice were examined following 5Gy, 10Gy, 15Gy and 20Gy doses of Gamma irradiation. The mice were killed 24 hours after the initial radiation exposure. Six mice were used for each irradiation dose.

iii. Tumour specimens

Samples 0.8cm in length, of six colonic adenocarcinomas were used for the purpose of this study. The samples were taken from fresh gross resection samples sent to the Histopathology Department for examination and subsequent diagnosis. The reason for examining tumour samples in this radiation study has been previously explained in the rationale of the research (page 60).

10. TISSUE PREPARATION for the SCANNING ELECTRON MICROSCOPE

Paraffin sections of control and irradiated small intestine of mouse and human tumour tissue were cut at $4\mu\text{m}$, mounted on glass coverslips and dried in a 37°C oven overnight. The sections were dewaxed in xylene, hydrated and stained by the modified AgNOR technique. They were subsequently dehydrated in 74 O.P. alcohol and air dried before mounting onto 25mm aluminium stubs. The sections were carbon coated using a bio-rad E6200 turbo coater and viewed in a Jeol JSM-840 scanning electron microscope operated in the back-scattered mode at between 3 and 20KV.

Part 3.

RESULTS

1. MEASUREMENT of the mean AgNOR AREA/NUCLEUS within the various COMPARTMENTS of CONTROL MOUSE SMALL INTESTINE (jejunum)

Controls

There was variation in the mean AgNOR area/nucleus within the various compartments of the jejunum samples (table 1, page 68). Within the epithelial compartment, the crypt epithelial cells exhibited the highest mean AgNOR area/nucleus, ie: $1.22\mu\text{m}^2$ (figure 6, page 75). The mean AgNOR area/nucleus was $0.55\mu\text{m}^2$ in the villus lamina propria, $0.53\mu\text{m}^2$ in the lower villus, $0.52\mu\text{m}^2$ in the muscle externa and $0.5\mu\text{m}^2$ in the lamina propria. The lowest mean AgNOR area/nucleus was found in the upper villus compartment, ie: $0.44\mu\text{m}^2$.

Shams

There was no significant difference in mean AgNOR area/nucleus between sham and control animals in any of the compartments studied (table 1, page 68).

TABLE 1.

	<u>AgNOR AREA/NUCLEUS</u>		<u>SHAM</u>
	<u>CONTROLS</u>		
	RANGE	MEAN	
Upper Villus	$0.40\mu\text{m}^2 - 0.48\mu\text{m}^2$	$0.44\mu\text{m}^2$	$0.46\mu\text{m}^2$
Lower Villus	$0.49\mu\text{m}^2 - 0.57\mu\text{m}^2$	$0.53\mu\text{m}^2$	$0.50\mu\text{m}^2$
Cryptal Epithelium	$1.17\mu\text{m}^2 - 1.27\mu\text{m}^2$	$1.22\mu\text{m}^2$	$1.25\mu\text{m}^2$
Villus Lamina Propria	$0.49\mu\text{m}^2 - 0.61\mu\text{m}^2$	$0.55\mu\text{m}^2$	$0.51\mu\text{m}^2$
Lamina Propria	$0.46\mu\text{m}^2 - 0.56\mu\text{m}^2$	$0.51\mu\text{m}^2$	$0.50\mu\text{m}^2$
Muscle Externa	$0.48\mu\text{m}^2 - 0.55\mu\text{m}^2$	$0.52\mu\text{m}^2$	$0.52\mu\text{m}^2$

2. MEASUREMENT of the mean AgNOR AREA/NUCLEUS within the various COMPARTMENTS of POST-IRRADIATED MOUSE SMALL INTESTINE (jejunum)

Treated Animals - Upper Villus Epithelium

Following irradiation of the upper villus epithelium with a 5Gy dose of gamma radiation, the mean AgNOR area/nucleus stayed within the control range from the 30 minute to the 72 hours time point with some fluctuation (graph 1, page 88). Results following 10Gy of gamma radiation showed a nonsignificant decrease in AgNOR area/nucleus between 6 and 12 hours. The mean AgNOR area/nucleus remained just below that of the control range for the 12, 24 and 72 hour time points (graph 2, page 89). After 15Gy of gamma radiation a gradual increase in AgNOR area/nucleus was seen between 30 minutes to 12 hours followed by a decrease between 12 and 72 hours. These variations in AgNOR area/nucleus were not significant (graph 3, page 90). Following 20Gy of gamma radiation the AgNOR area/nucleus displayed an initial decrease between 30 minutes and 6 hours followed by a nonsignificant increase to slightly above the upper limit of the control range at 12 hours and remained at that level for the 24 and 72 hour time points (graph 4, page 91).

Treated Animals - Lower Villus Epithelium

Following irradiation of the lower villus with a 5Gy dose of gamma radiation there was a nonsignificant decrease in AgNOR area/nucleus between 30 minutes and 6 hours post irradiation. There upon the mean AgNOR area/nucleus remained within the control range at the 12, 24 and 72 hour time points (graph 5, page 92). After 10Gy of gamma radiation all mean AgNOR area/nucleus values were above that of the control range. Increases were significantly above control values at 30 minutes ($p=0.0531$, Vol II, page 7), 6 hours ($p=0.0001$, Vol II, page 19) and 24 hours ($p=0.0001$, Vol II, page 43) post irradiation (graph 6, page 93). Following

15Gy of gamma radiation there were small nonsignificant decreases and increases, all of which were within the control range (graph 7, page 94). Results from the 20Gy dose of gamma radiation showed that the AgNOR area/nucleus increased significantly between 30 minutes ($p=0.0531$, Vol II, page 7) and 72 hours ($p=0.0001$, Vol II, page 55) with its maximum value at 12 hours ($p=0.0001$, Vol II, page 31), (graph 8, page 95).

Treated Animals - Cryptal Epithelium

Following irradiation of the crypt epithelium with a 5Gy dose of gamma radiation, the AgNOR area/nucleus increased significantly between the 30 minute ($p=0.0001$, Vol II, page 9) and 6 hour time points ($p=0.0001$, Vol II, page 21). This was followed by a drop in values to the control range at the 72 hour time point ($p=0.0001$, Vol II, page 57), (graph 9, page 96). The results following 10Gy of gamma radiation show a significant increase in mean AgNOR area/nucleus at 30 minutes post irradiation ($p=0.0001$, Vol II, page 9). The values from the remainder of the time points up to 24 hours showed further significant increases followed by a slight decline at 72 hours ($p=0.0001$, Vol II, page 57), (graph 10, page 97). After 15Gy of gamma radiation there was a significant increase in the mean AgNOR area/nucleus recorded at 30 minutes ($p=0.0001$, Vol II, page 9) followed by a substantial increase at 6 hours ($p=0.0001$, Vol II, page 21). This was followed by further significant rises up to 72 hours post irradiation ($p=0.0001$, Vol II, page 57), (graph 11, page 98). Following 20Gy of gamma radiation the mean AgNOR area/nucleus at the 30 minute time point ($p=0.0001$, Vol II, page 9) displayed the largest initial increase in comparison to all other results from all compartments at all doses. For the subsequent time points of 6, 12, and 24 hours the AgNOR area/nucleus increased notably to a maximum of $2.7\mu\text{m}^2$. Thereupon the value decreased at 72 hours ($p=0.0001$, Vol II, page 57) post irradiation but was still significantly above the control range (graph 12, page 99).

Treated Animals - Villus Lamina Propria

Following irradiation of the villus lamina propria with a 5Gy dose of gamma radiation all results stayed within the control range at all time points (graph 13, page 100). After 10Gy of gamma radiation all results were within or just below the control range (graph 14, page 101). Following 15Gy of gamma radiation the mean AgNOR area/nucleus initially stayed within the control range up to the 6 hour time point and fell below the control range at the 12 hour time point where it remained until the 24 and 72 hour time points (graph 15, page 102). After 20Gy of gamma radiation the mean AgNOR area/nucleus at 30 minutes post irradiation was within the control range. At 6 hours it had increased to just above the control range but then dropped back into the control range for the 12, 24 and 72 hour time points (graph 16, page 103).

Treated Animals - Lamina Propria

Following irradiation of the lamina propria with a 5Gy dose of gamma radiation, the mean AgNOR area/nucleus stayed within the control range except at the 72 hour time point where it showed a significant increase ($p=0.0001$, Vol II, page 61), (graph 17, page 104). Following 10Gy of gamma radiation all results remained within the control range (graph 18, page 105). Following 15Gy of gamma radiation the mean AgNOR/nucleus remained just below or within the control range (graph 19, page 106). Following 20Gy of gamma radiation the mean AgNOR area/nucleus at 30 minutes post irradiation was just above the control range. It fell within the control range at 6 hours and subsequently increased to just above at 24 hours post irradiation with a return to the control band at 72 hours (graph 20, page 107).

Treated Animals - Muscle Externa

Following irradiation of the muscle externa with a 5Gy dose of gamma radiation, the mean AgNOR area/nucleus showed a significant increase from being within the control range at 30 minutes to above at

12 hours ($p=0.0003$, Vol II, page 39). This was followed by a sharp decrease at 24 hours post irradiation and a mean AgNOR area/nucleus within the control range at 72 hours (graph 21, page 108). After 10Gy of gamma radiation all results were within the control range (graph 22, page 109). After 15Gy of gamma radiation the mean AgNOR area/nucleus at the 30 minute time point was just above the control range. It dropped but was still within the control range at 6 hours and continued to show decline until being below that of the control range at 72 hours post irradiation (graph 23, page 110). After 20Gy of gamma radiation the mean AgNOR area/nucleus was significantly below the control range at 30 minutes ($p=0.0001$, Vol II, page 15). At the 6 hour ($p=0.0026$, Vol II, page 27) and 12 hour ($p=0.0003$, Vol II, page 39) time points there was a significant increase in mean AgNOR area/nucleus and it remained significantly above the control range at 24 hours ($p=0.0001$, Vol II, page 51) and 72 hours ($p=0.0022$, Vol II, page 63) post irradiation (graph 24, page 111).

3. INVESTIGATION into the POSSIBILITY of a RELATIONSHIP between mean AgNOR AREA/NUCLEUS and mean AgNOR NUMBER/NUCLEUS in POST-IRRADIATED SAMPLES of MOUSE SMALL INTESTINE (graphs 25-48)

The compartments and graphs studied were as follows:

- upper villus (graphs 25-28, pages 112-115)
- lower villus (graphs 29-32, pages 116-119)
- cryptal epithelium (graphs 33-36, pages 120-123)
- villus lamina propria (graphs 37-40, pages 124-127)
- lamina propria (graphs 41-44, pages 128-131)
- muscle externa (graphs 45-48, pages 132-135)

Graphs of the mean AgNOR area/nucleus and mean AgNOR number/nucleus following various doses of gamma radiation ranging from

5Gy to 20Gy were reviewed and compared. With the exception of the cryptal epithelium, it was determined that, there was no specific relationship between the two parameters in any of the compartments examined.

Within the cryptal epithelial compartment (graphs 33-36, pages 120-123), however, there appeared to be a significant inverse relationship between mean AgNOR area/nucleus and mean AgNOR number/nucleus, post-irradiation. This inverse relationship manifested itself as a decrease in AgNOR number/nucleus and an increase in AgNOR area/nucleus. On examination of the data the relationship appeared to be both dose and post irradiation time dependent (graphs 34-36, pages 121-123). From Graph 36, page 123, it was seen that the most significant increase in mean AgNOR area/nucleus and decrease in mean AgNOR number/nucleus occurred after the 20Gy dose between 24 and 72 hours. The smallest increase in AgNOR area and decrease in AgNOR number occurred after the 10Gy dose (graph 34, page 121). Following 5Gy of radiation (graph 33, page 120), the inverse relationship was not so pronounced. The area increased almost immediately but then fell to within the control range whereas the numbers also showed an initial increase but then followed the same pattern as with the other doses and began a steady decline.

4. The OBSERVATION of the STRUCTURE of SILVER-STAINED NUCLEOLAR ORGANISER REGION ASSOCIATED PROTEINS in CONTROL, IRRADIATED and TUMOUR TISSUE using the SCANNING ELECTRON MICROSCOPE (standard emission mode & back-scattered mode)

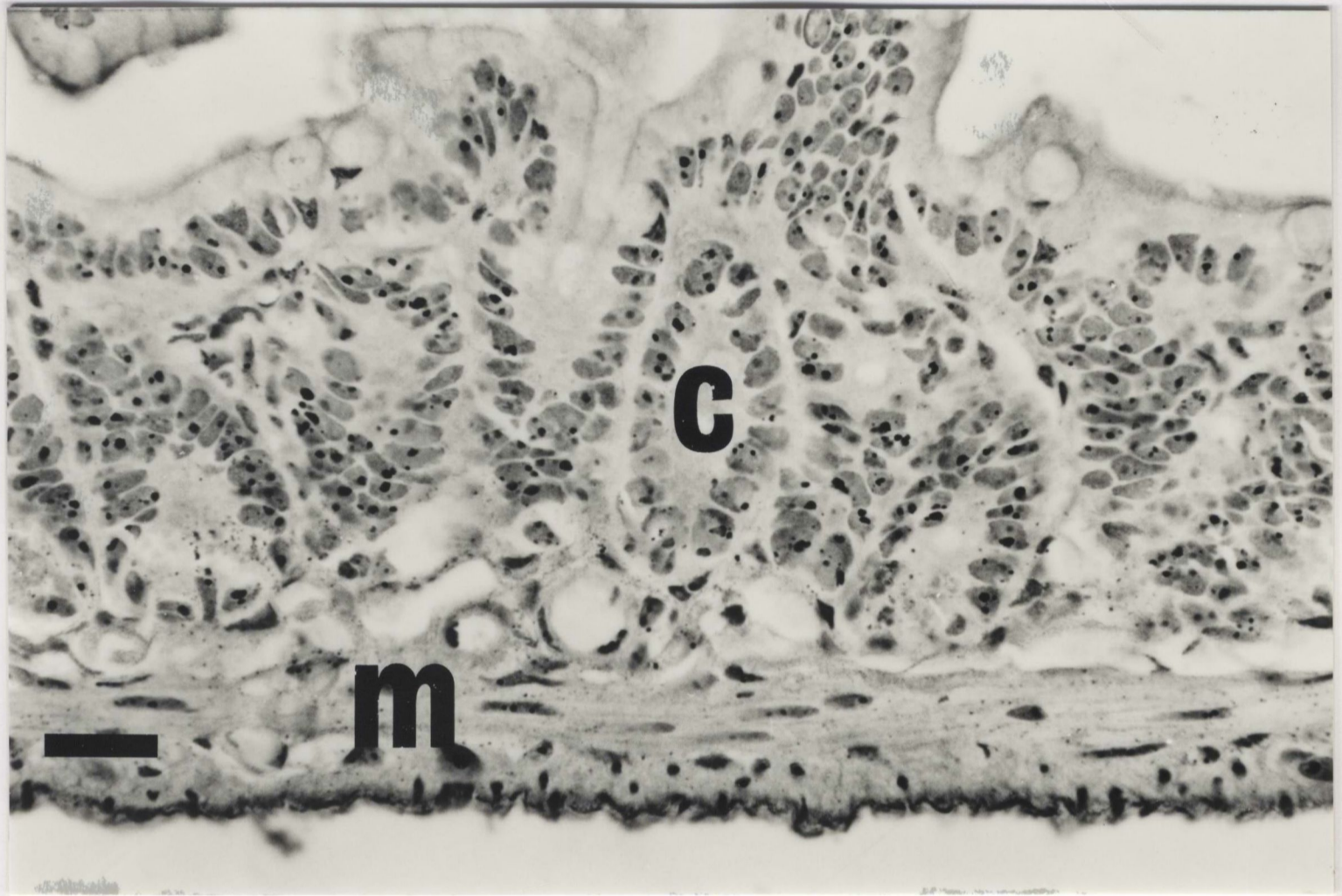
Using the scanning electron microscope (standard emission mode & back-scattered mode) silver-stained nucleolar organiser regions from control mouse small intestine appeared as uniform structures with rounded

edges (figures 7 & 8, pages 76 & 77). A comparative light microscopy photograph of a similar area in the crypt epithelium is shown in figure 9, page 78.

AgNORs from irradiated tissue also appeared as uniform structures with rounded edges (figure 10, page 79). A comparison of AgNORs in irradiated tissue (10Gy after 60 minutes) using a high power scanning electron microscope in the back-scattered mode and in the standard emission mode is demonstrated in figures 11 & 12, pages 80 & 81.

The AgNORs seen in tumour tissue using the scanning electron microscope appeared as bizarre structures resembling icebergs. A photograph showing the silver-stained NORs in standard emission mode is displayed in figure 13, page 82. The back-scattered mode of the same field is shown in figure 14, page 83. The inverse back-scattered mode is shown in figure 15, page 84. High power scanning electron microscope photographs of tumour AgNORs (adenocarcinoma) are shown in figures 16 & 17, pages 85 & 86. The peaks of these bizarre structures are clearly visible. An enlargement of one of these tumour AgNORs is shown in figure 18, page 87.

Figure 6: A paraffin section of control mouse small intestine (jejunum). AgNORs are seen as small black dots within the nucleus.



M = muscle

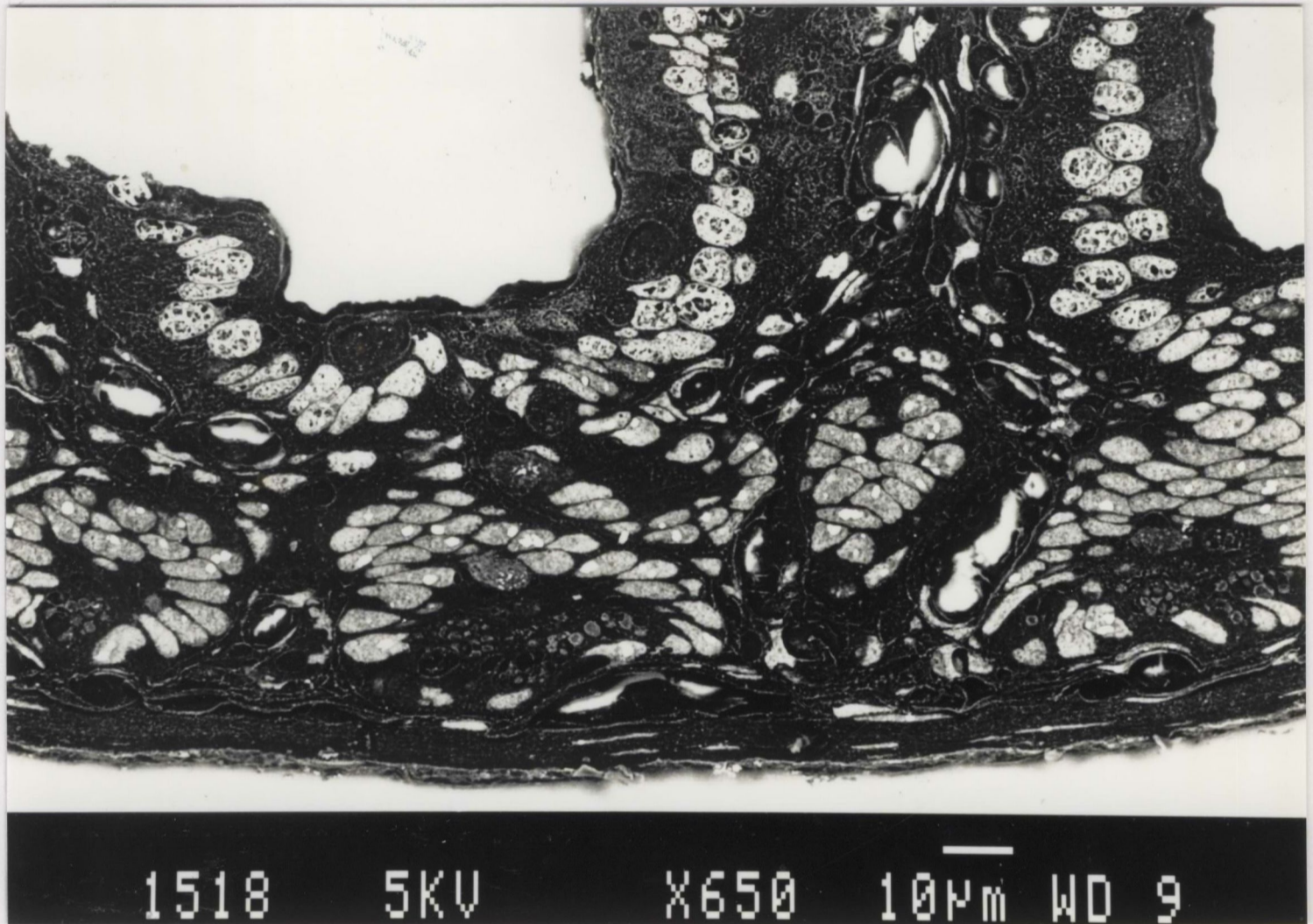
C = crypt

Figure 7: A paraffin section of control mouse small intestine as viewed by the scanning electron microscope (back-scattered mode), 5KV.



AgNORs are seen as white dots within the nucleus.

Figure 8: A paraffin section of control mouse small intestine as viewed by the scanning electron microscope (back-scattered mode), 5KV.



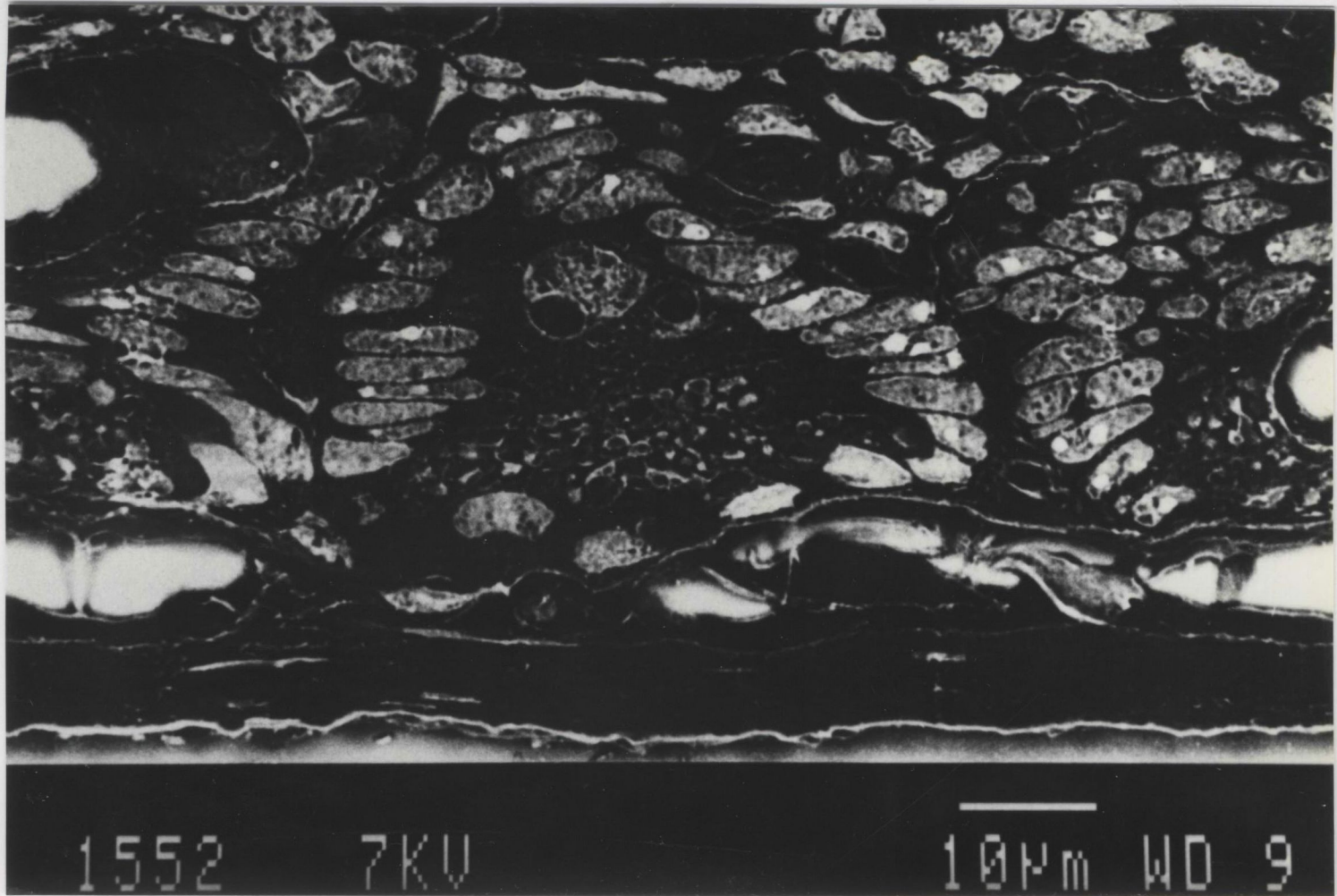
AgNORs are seen as white dots within the nucleus.

Figure 9: A light micrograph of a paraffin section of control mouse small intestine (jejunum) showing AgNORs within the cryptal epithelium compartment.



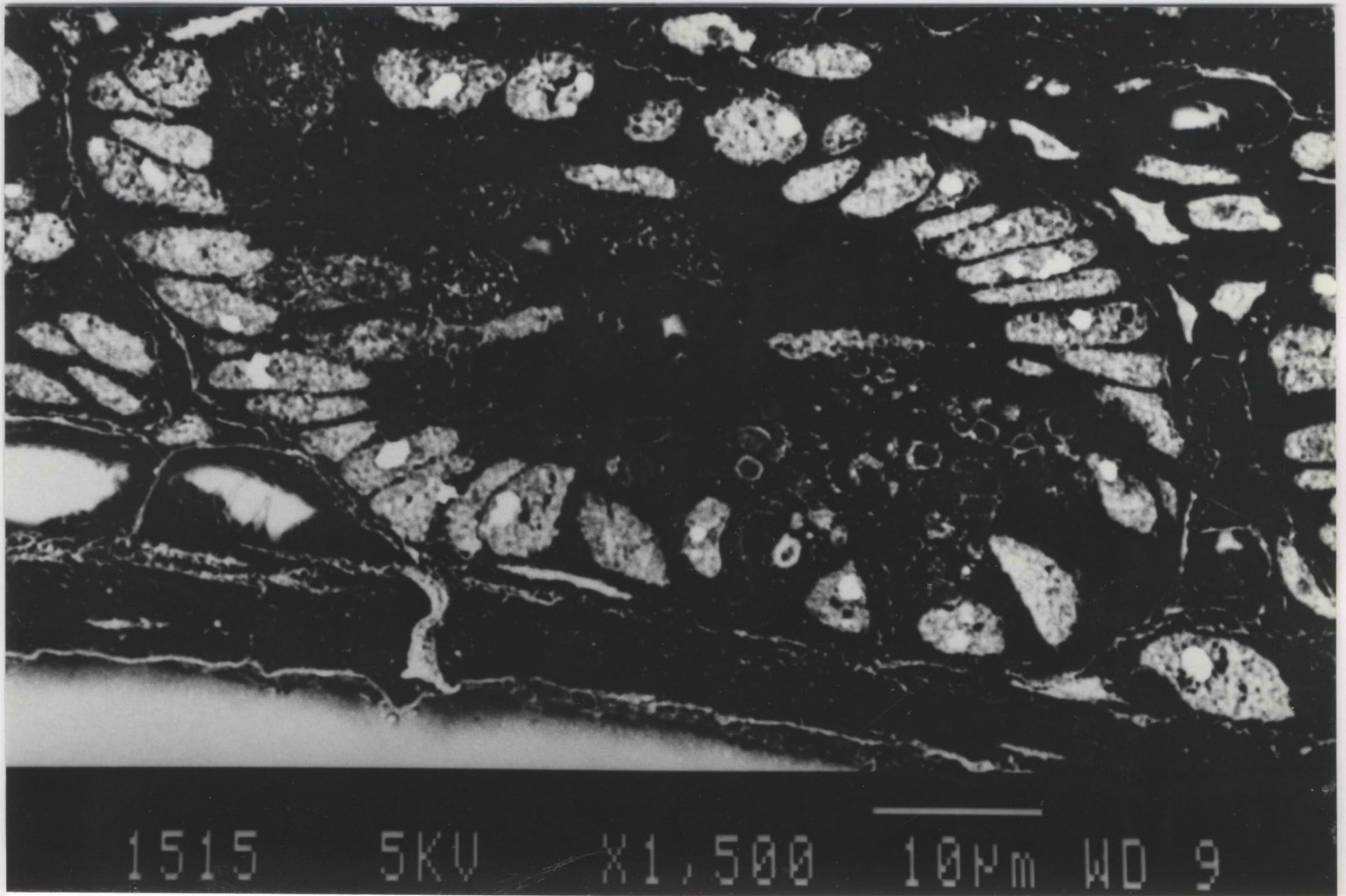
AgNORs are seen as small black dots within the nucleus.

Figure 10: A paraffin section of irradiated mouse small intestine (jejunum), 10Gy at 24 hours post irradiation, as viewed by the scanning electron microscope (back-scattered mode), 7KV.



AgNORs are seen as small white dots within the nucleus.

Figure 11: A paraffin section of irradiated mouse small intestine (jejunum), 15Gy at 6 hours post irradiation, as viewed by the scanning electron microscope in the back-scattered mode, 5KV.



AgNORs are seen as small white dots within the nucleus.

Figure 12: A paraffin section of irradiated mouse small intestine (jejunum crypt), 15Gy at 6 hours post irradiation, as viewed by the scanning electron microscope in the standard emission mode, 5KV.

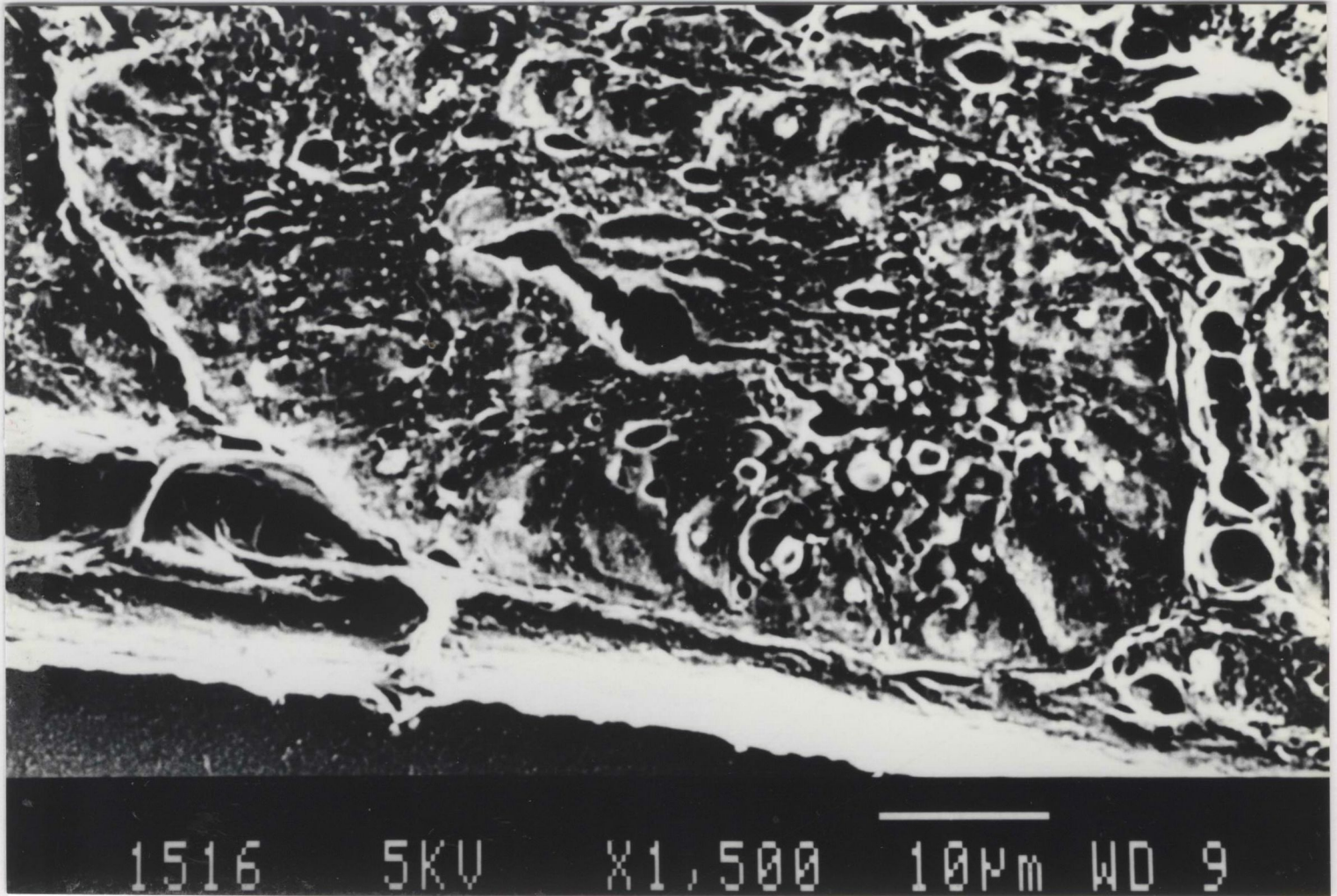
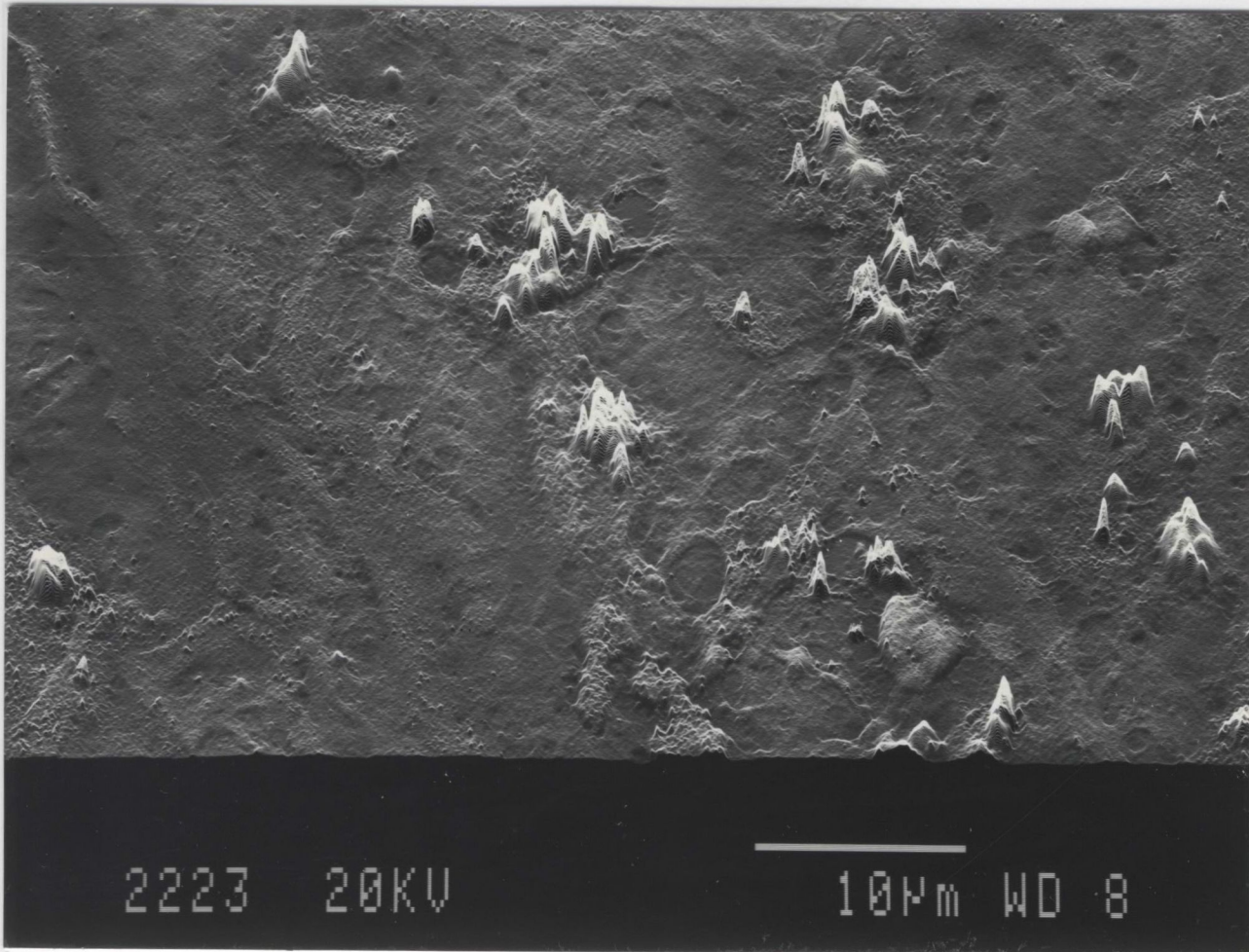
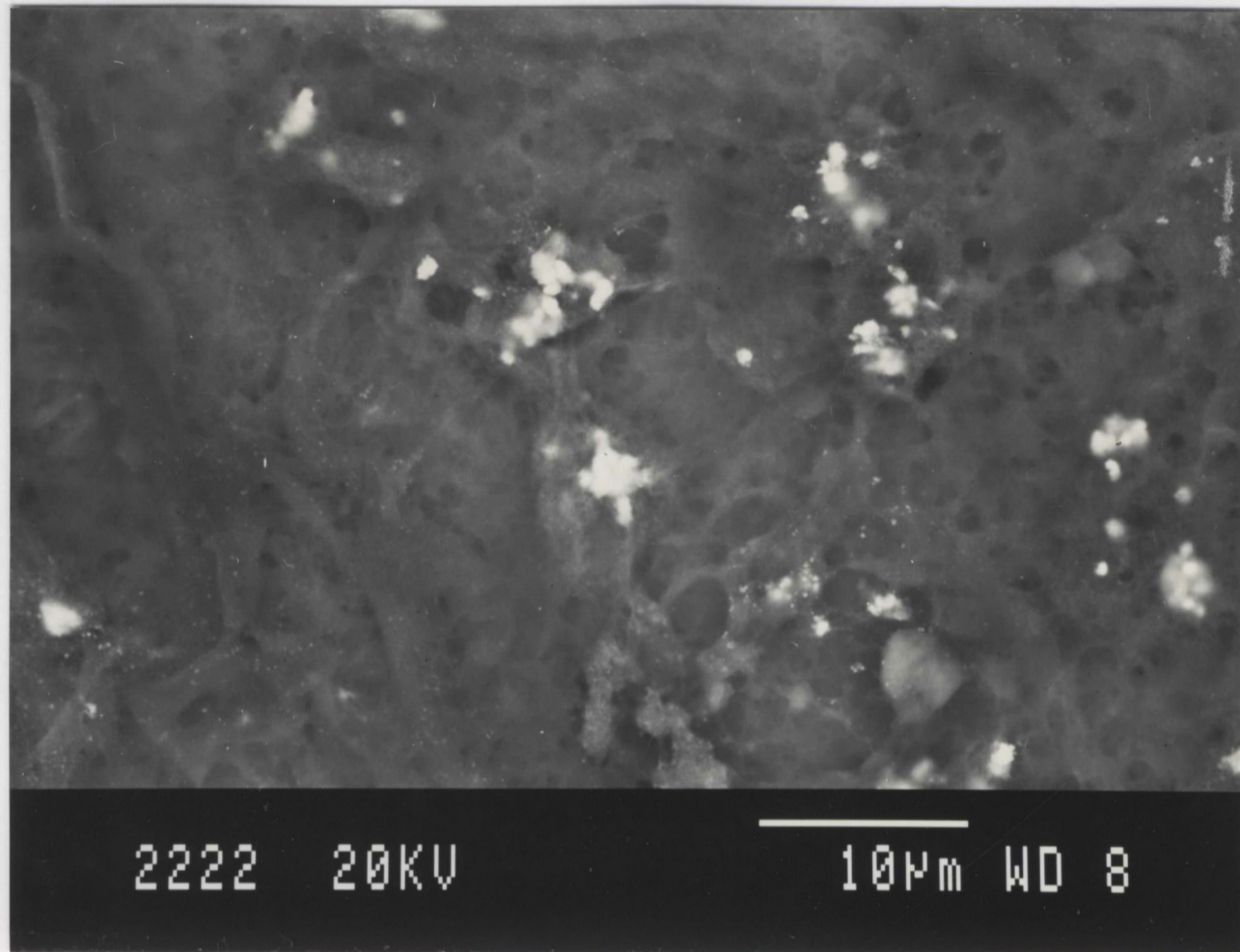


Figure 13: A paraffin section of human tumour tissue (adenocarcinoma) as viewed by the scanning electron microscope using the standard emission mode, 20KV.



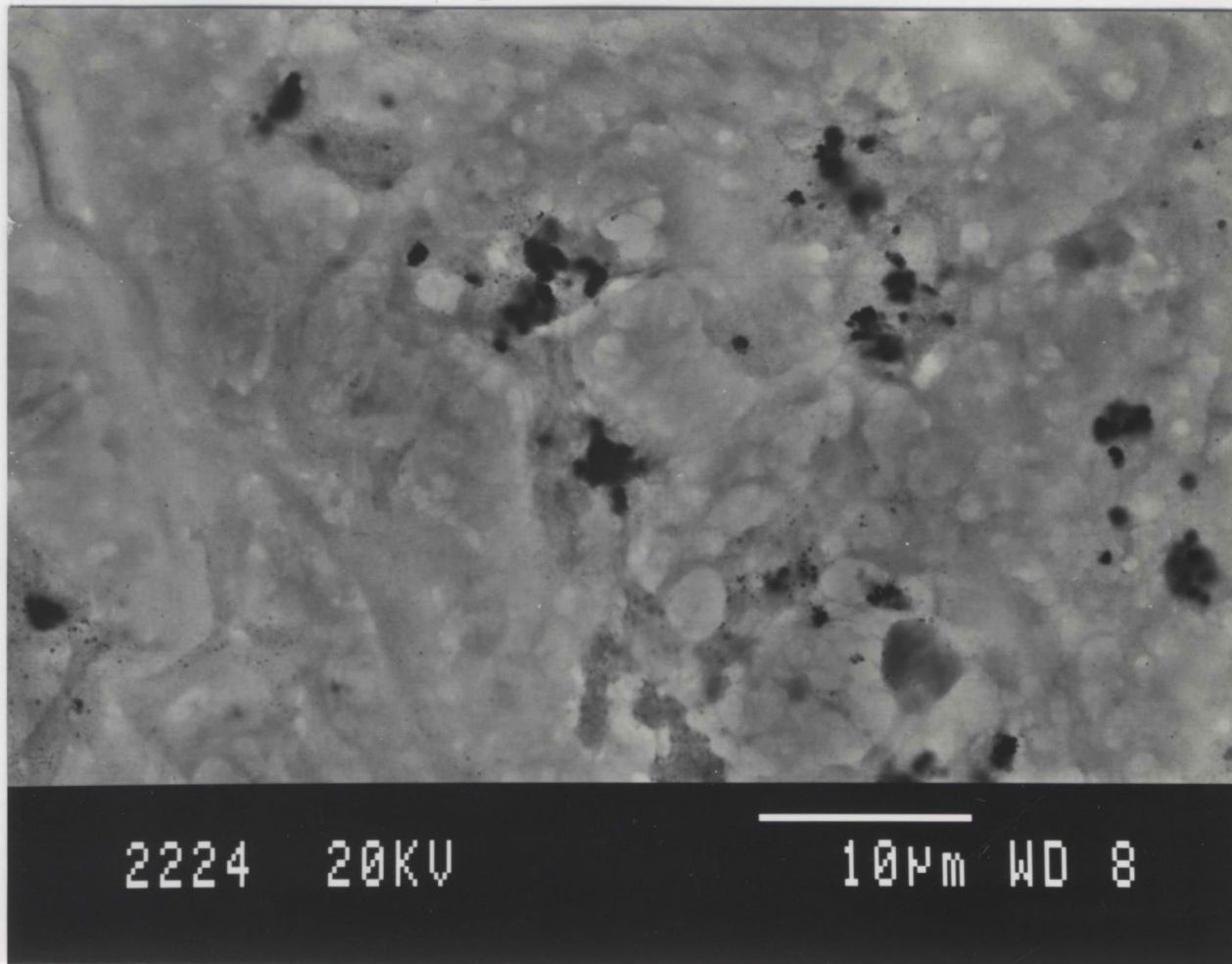
Tumour AgNORs are seen as bizarre, white, irregular structures.

Figure 14: A paraffin section of human tumour tissue (adenocarcinoma) as viewed by the scanning electron microscope using the back-scattered mode, 20KV.



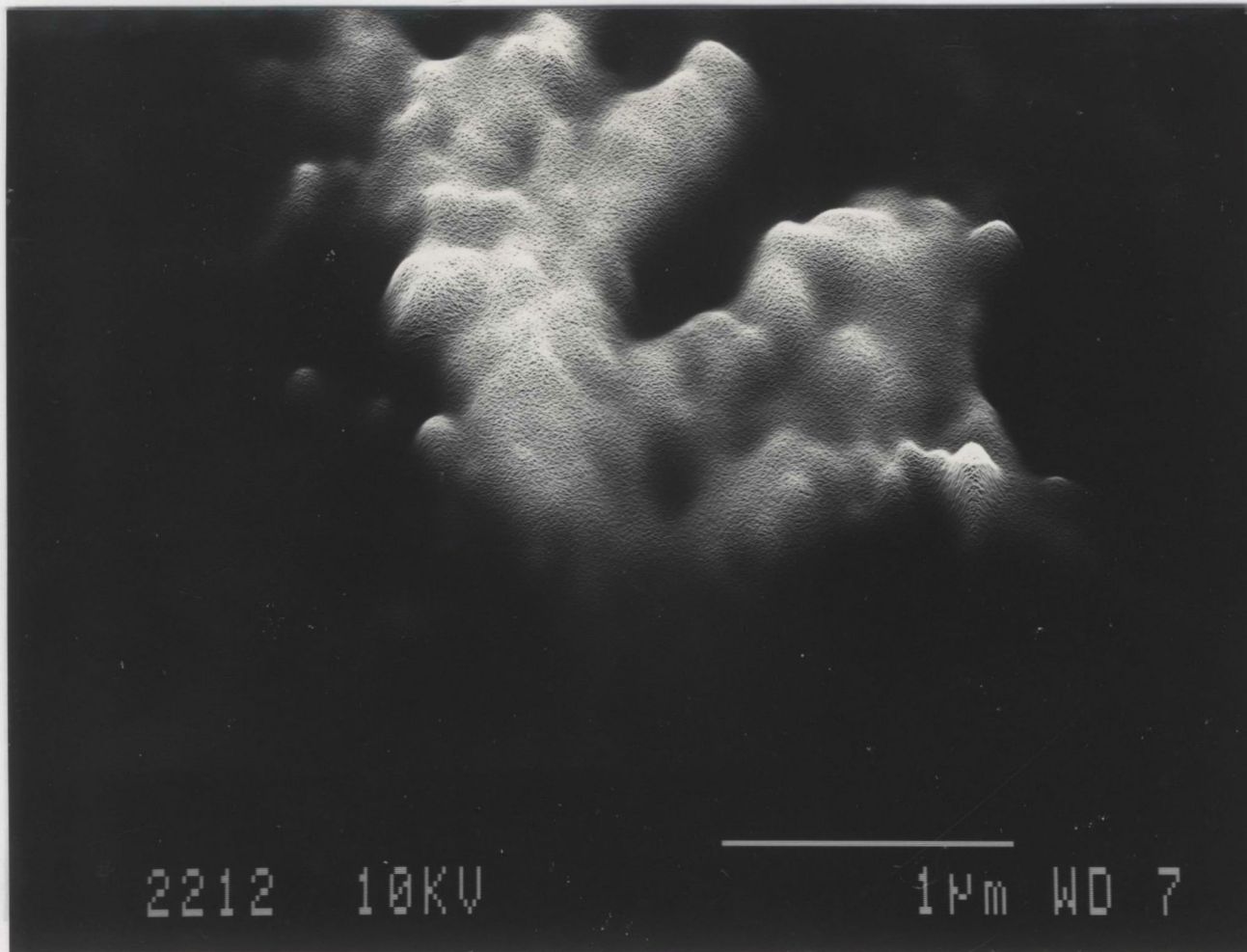
Tumour AgNORs are seen as irregular, white structures.

Figure 15: A paraffin section of human tumour tissue (adenocarcinoma) as viewed by the scanning electron microscope using the inverse back-scattered mode, 20KV.



Tumour AgNORs are seen as irregular, black structures.

Figure 16: A paraffin section of human tumour tissue (adenocarcinoma) as viewed by the scanning electron microscope using the back-scattered mode, 10KV.



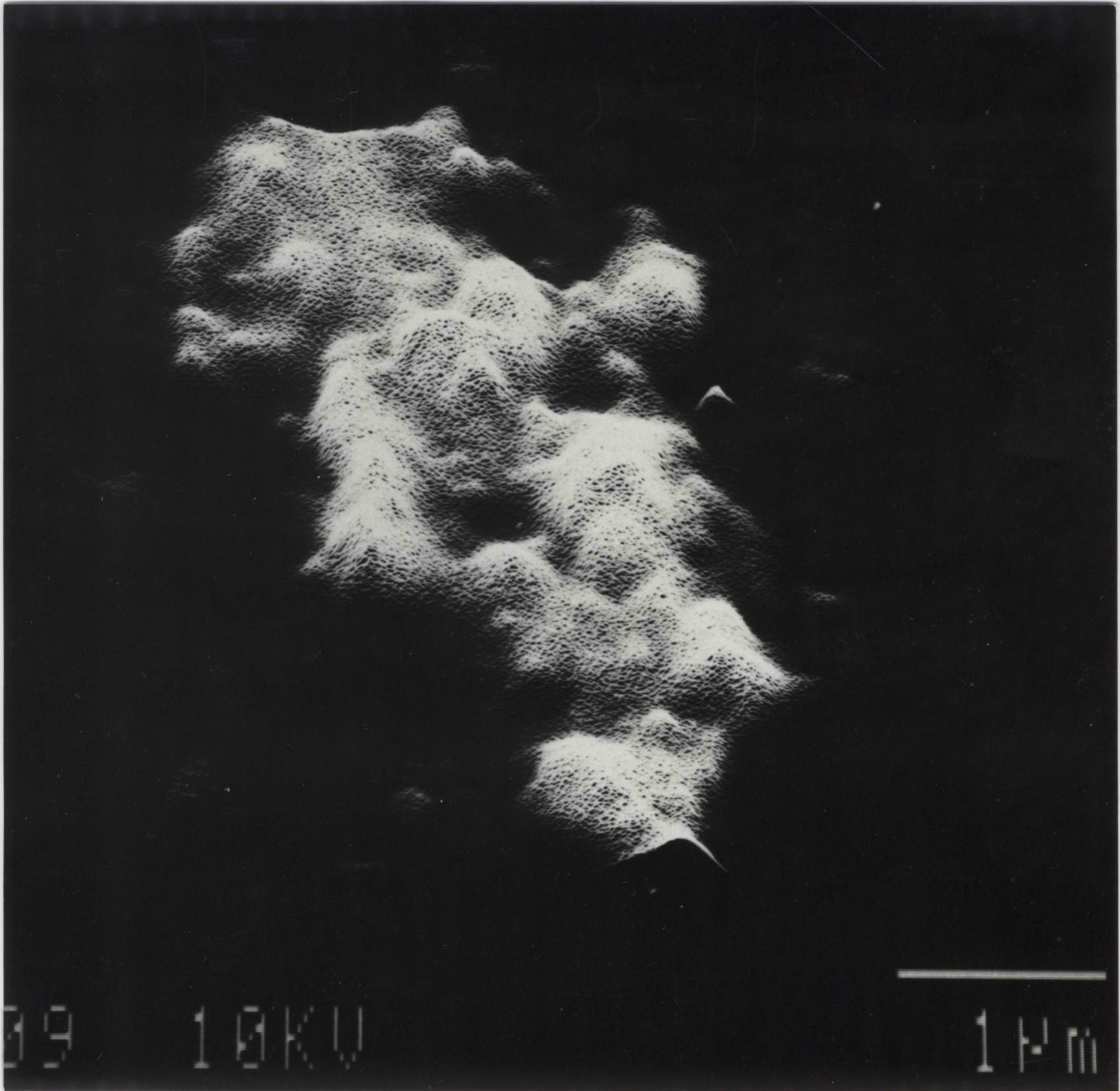
Tumour AgNORs are seen as bizarre, white, irregular structures.

Figure 17: A paraffin section of human tumour tissue (adenocarcinoma) as viewed by the scanning electron microscope using the back-scattered mode, 20KV.



Tumour AgNORs are seen as bizarre, white, irregular structures.

Figure 18: A paraffin section of human tumour tissue (adenocarcinoma) as viewed by the scanning electron microscope (back-scattered mode), using a high magnification, 10KV.

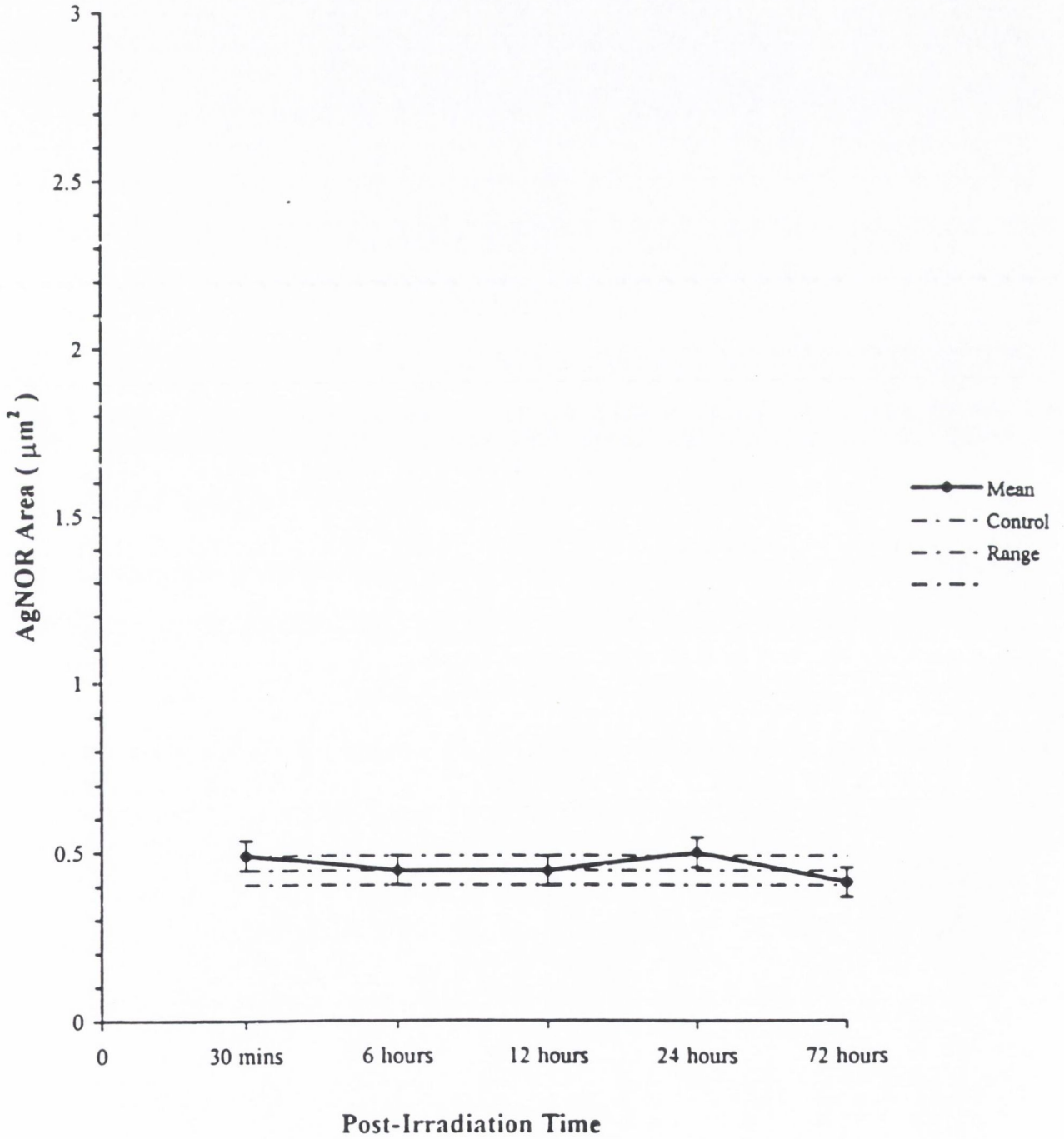


Tumour AgNORs are seen as bizarre, white, irregular structures resembling icebergs.

Graph 1

AgNOR Area vs Post-Irradiation Time *Upper Villus*

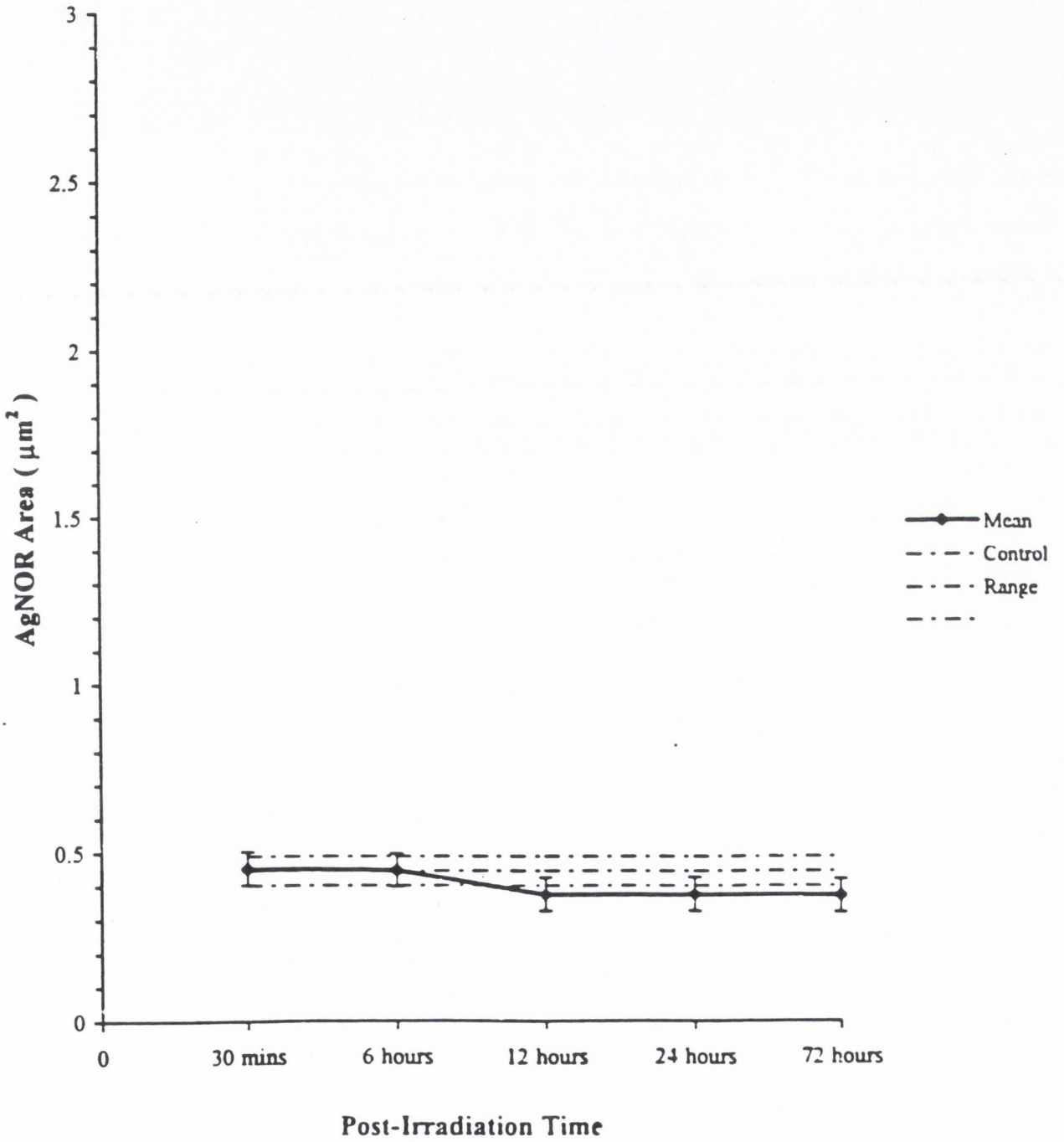
5Gy



Graph 2

AgNOR Area vs Post-Irradiation Time *Upper Villus*

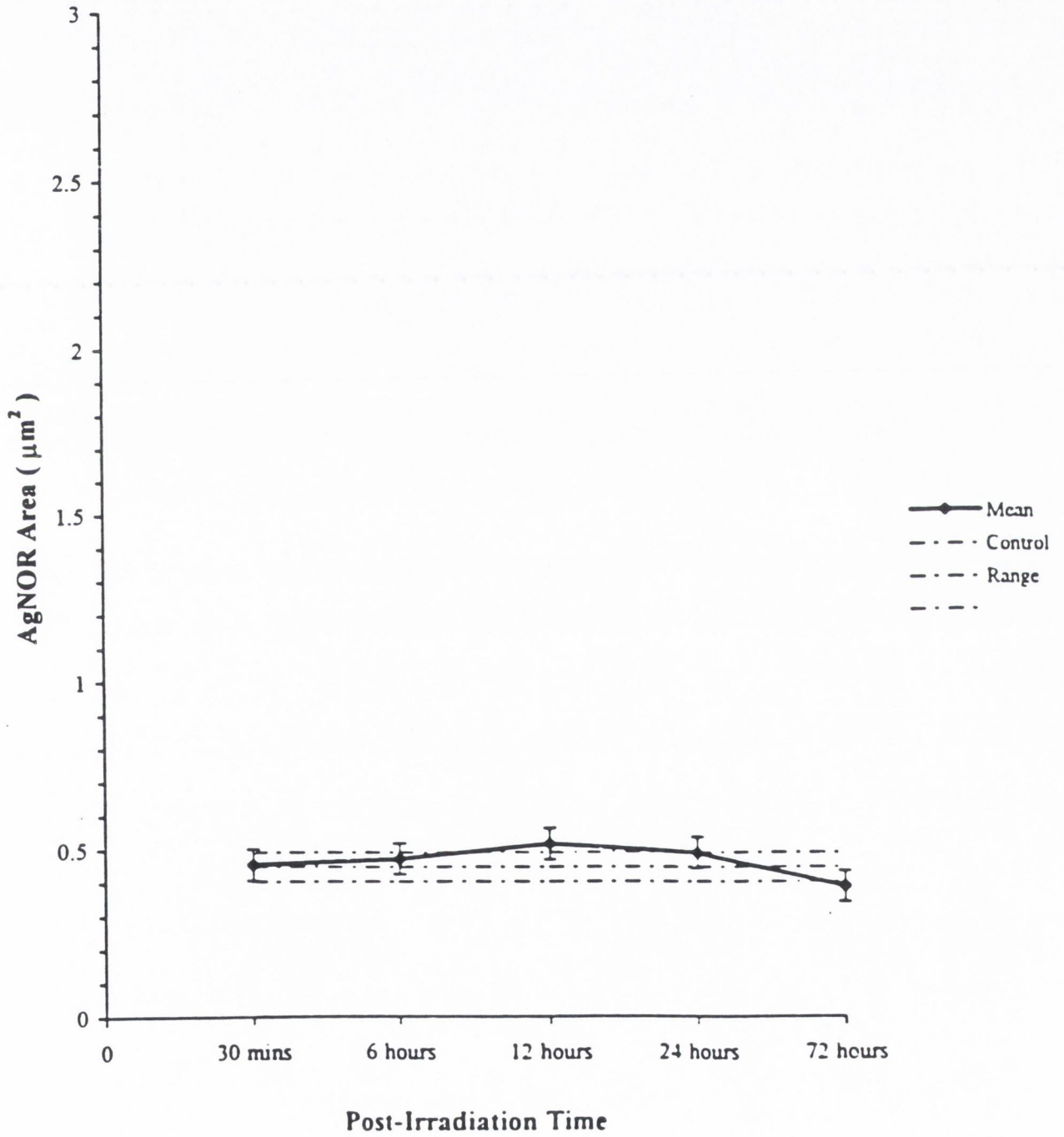
10Gy



Graph 3

AgNOR Area vs Post-Irradiation Time *Upper Villus*

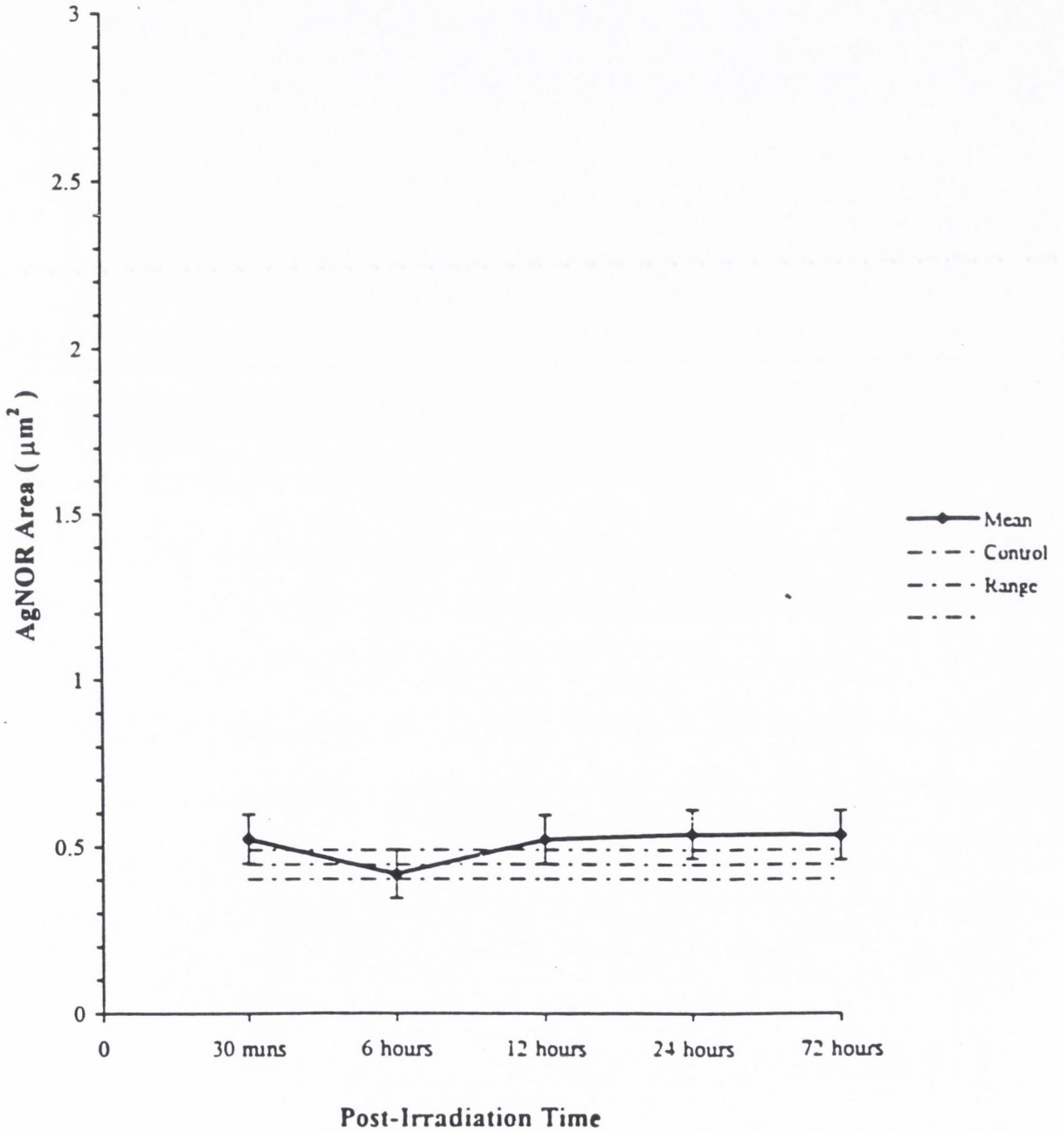
15Gy



Graph 4

AgNOR Area vs Post-Irradiation Time *Upper Villus*

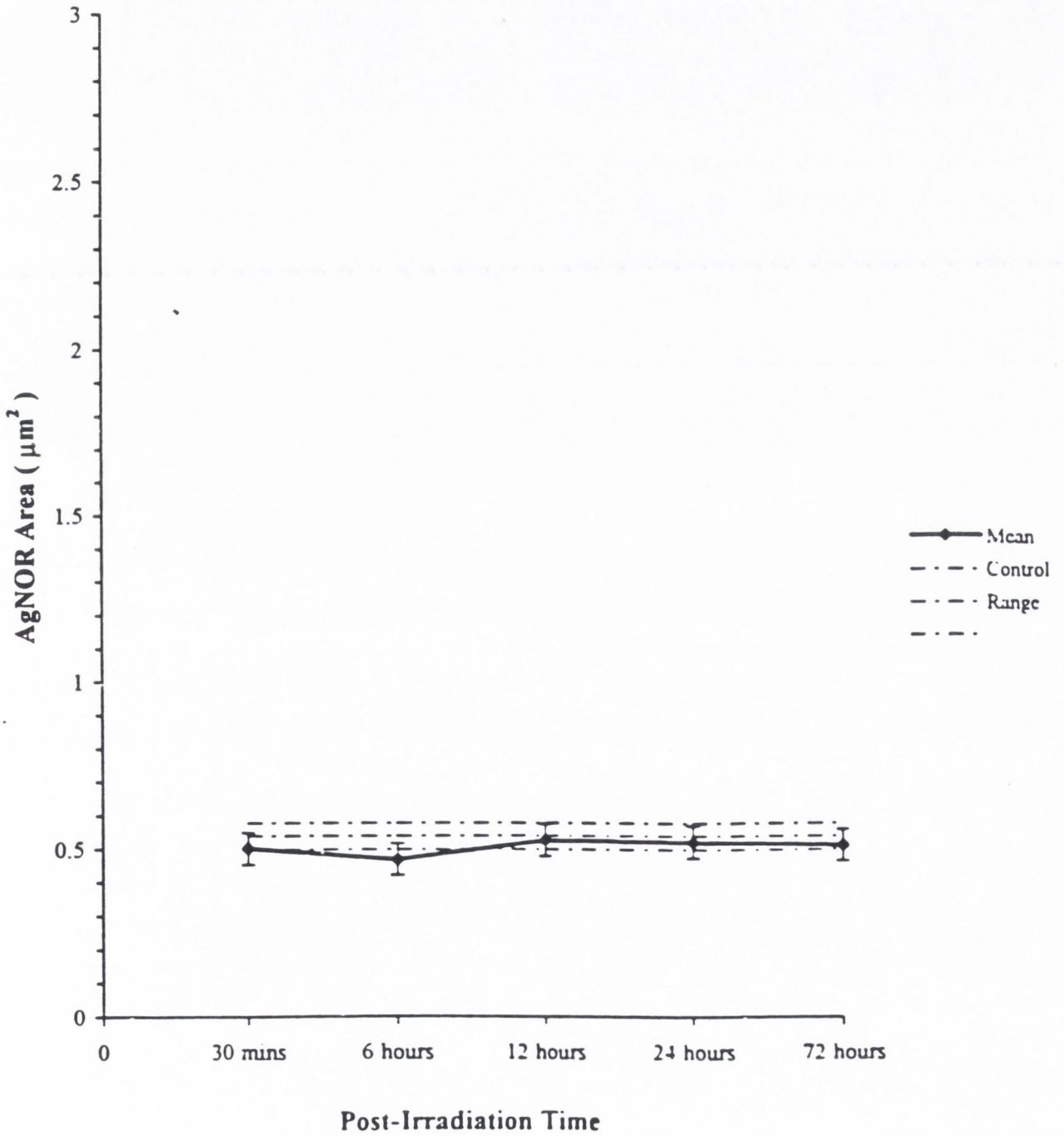
20Gy



Graph 5

AgNOR Area vs Post-Irradiation Time *Lower Villus*

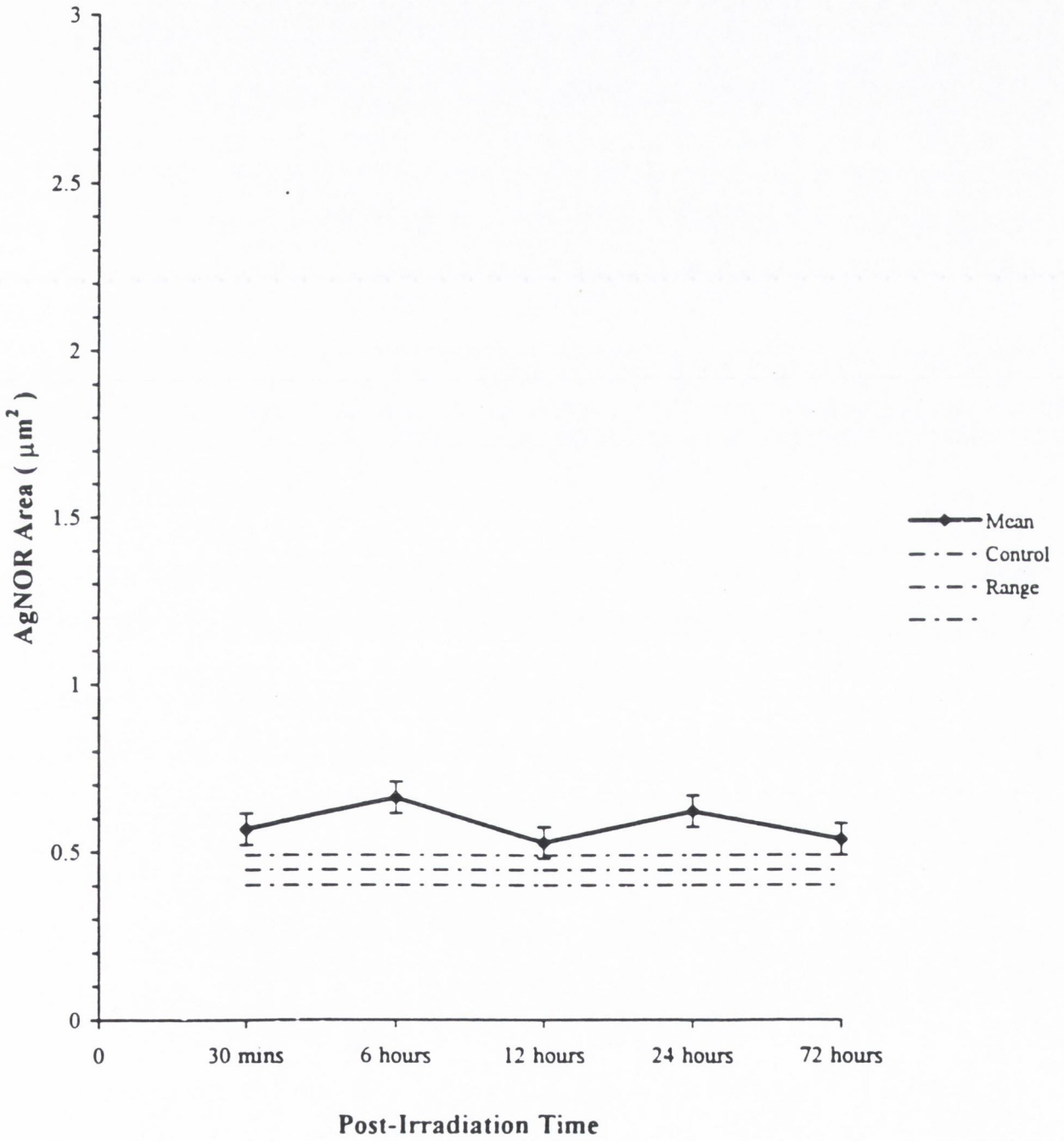
5Gy



Graph 6

AgNOR Area vs Post-Irradiation Time *Lower Villus*

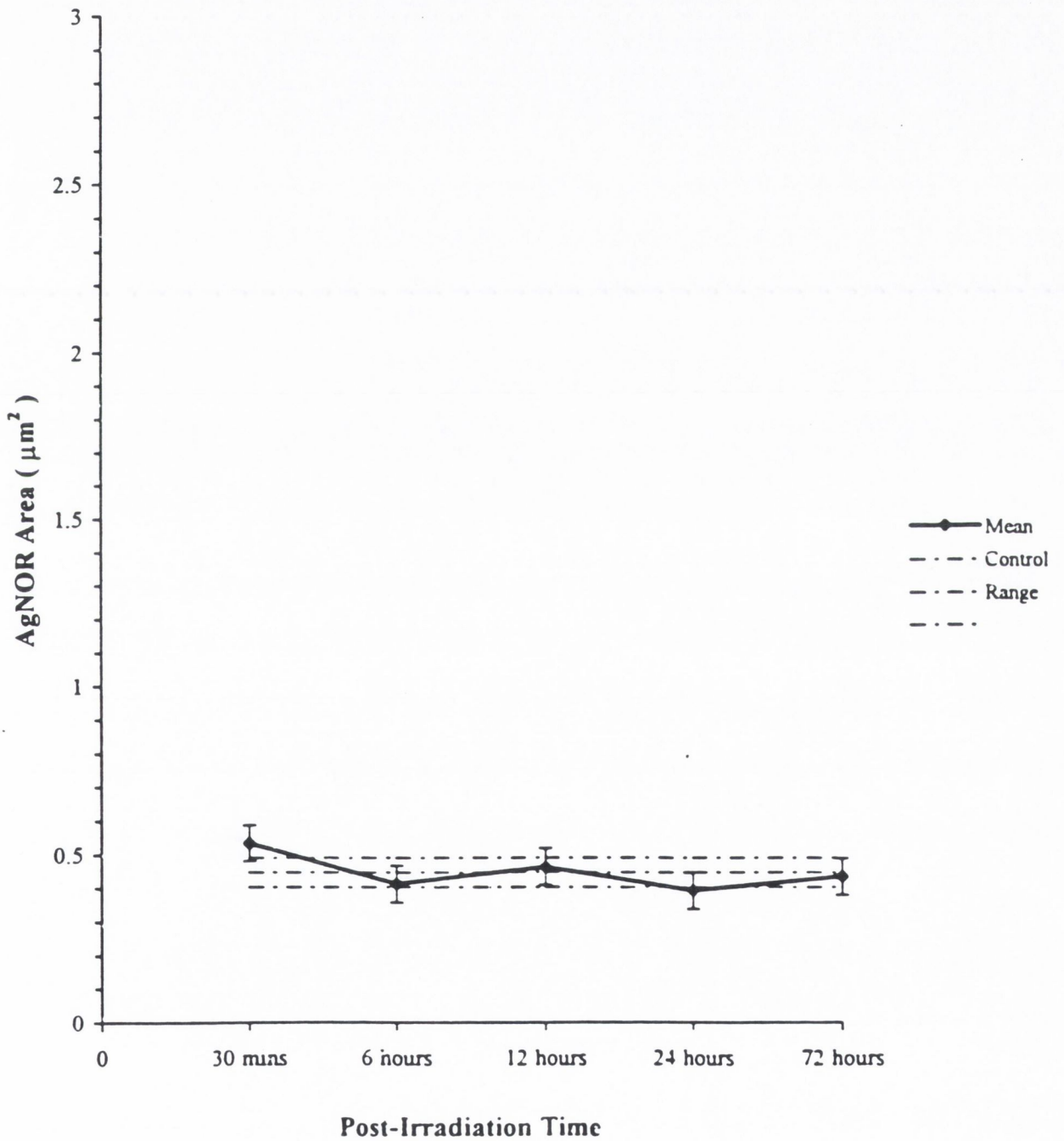
10Gy



Graph 7

AgNOR Area vs Post-Irradiation Time *Lower Villus*

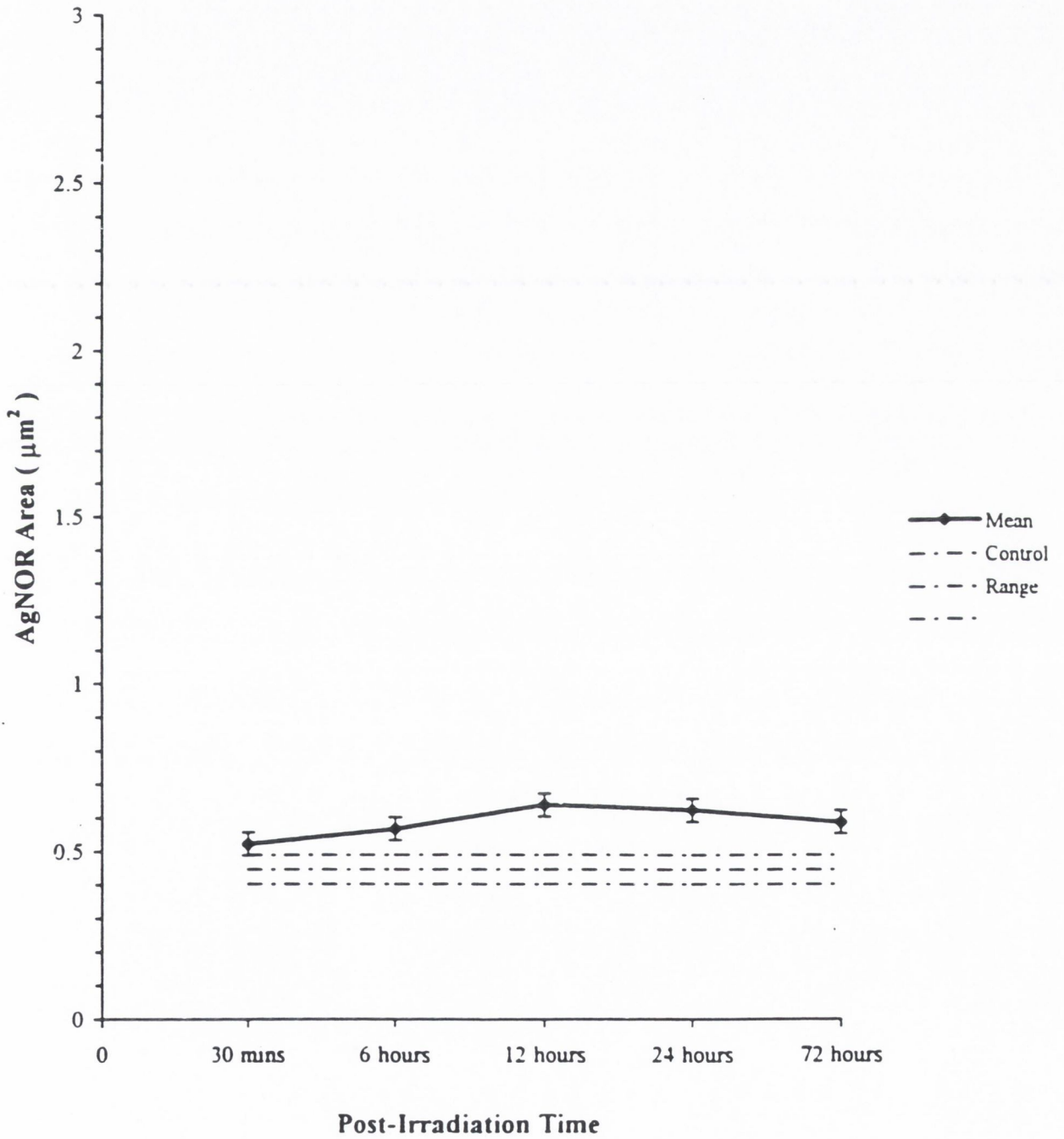
15Gy



Graph 8

AgNOR Area vs Post-Irradiation Time *Lower Villus*

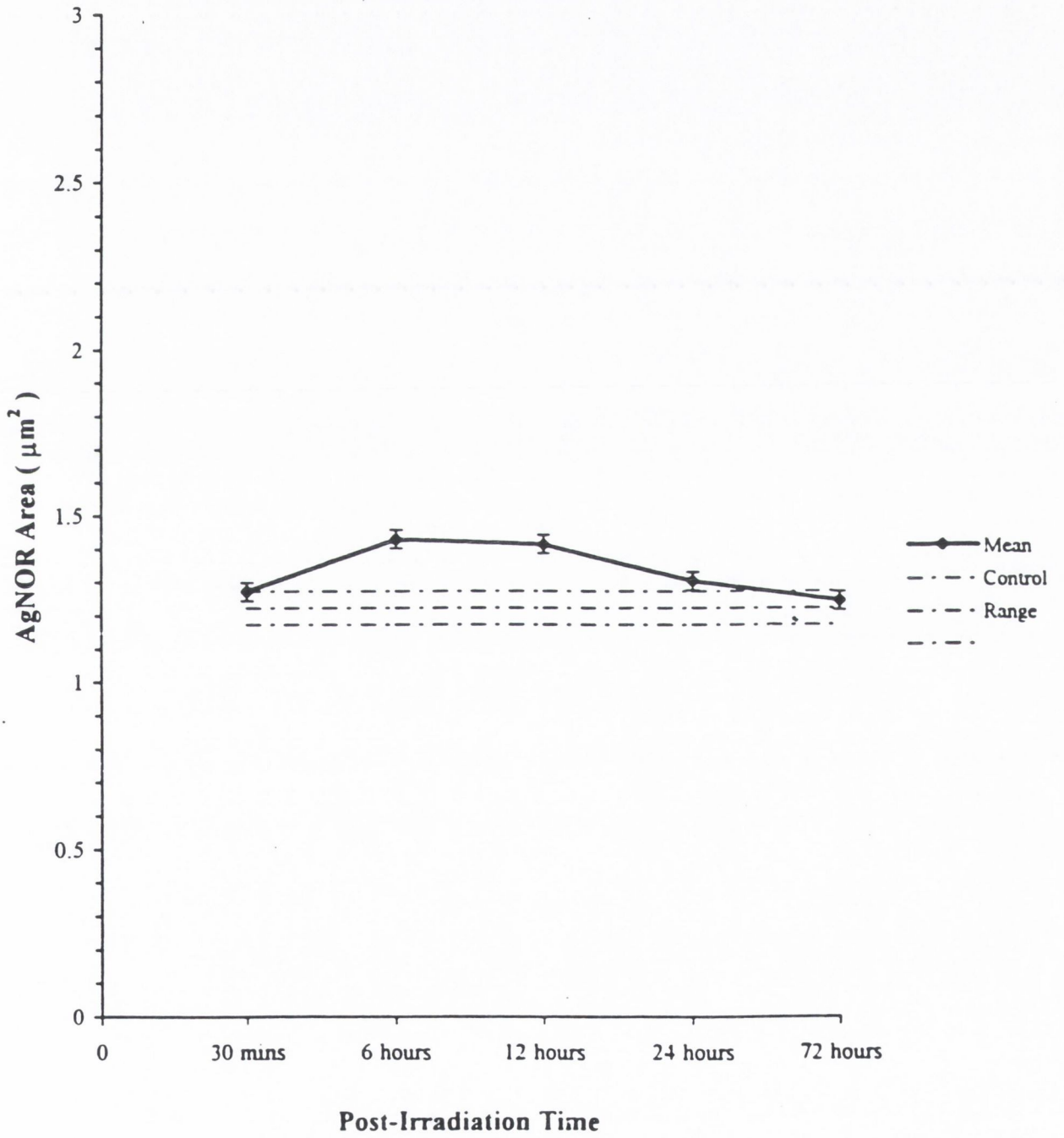
20Gy



Graph 9

AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

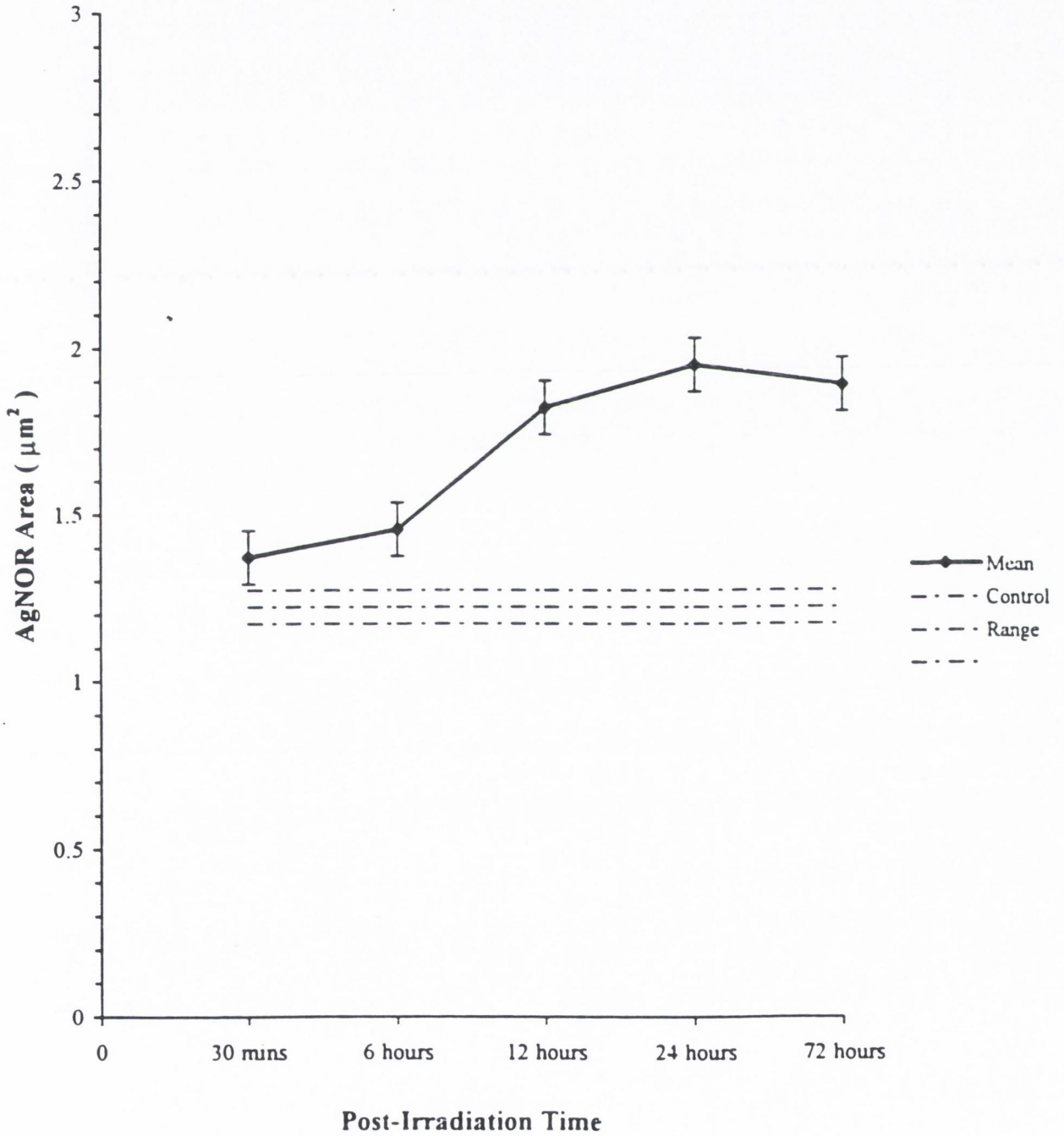
5Gy



Graph 10

AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

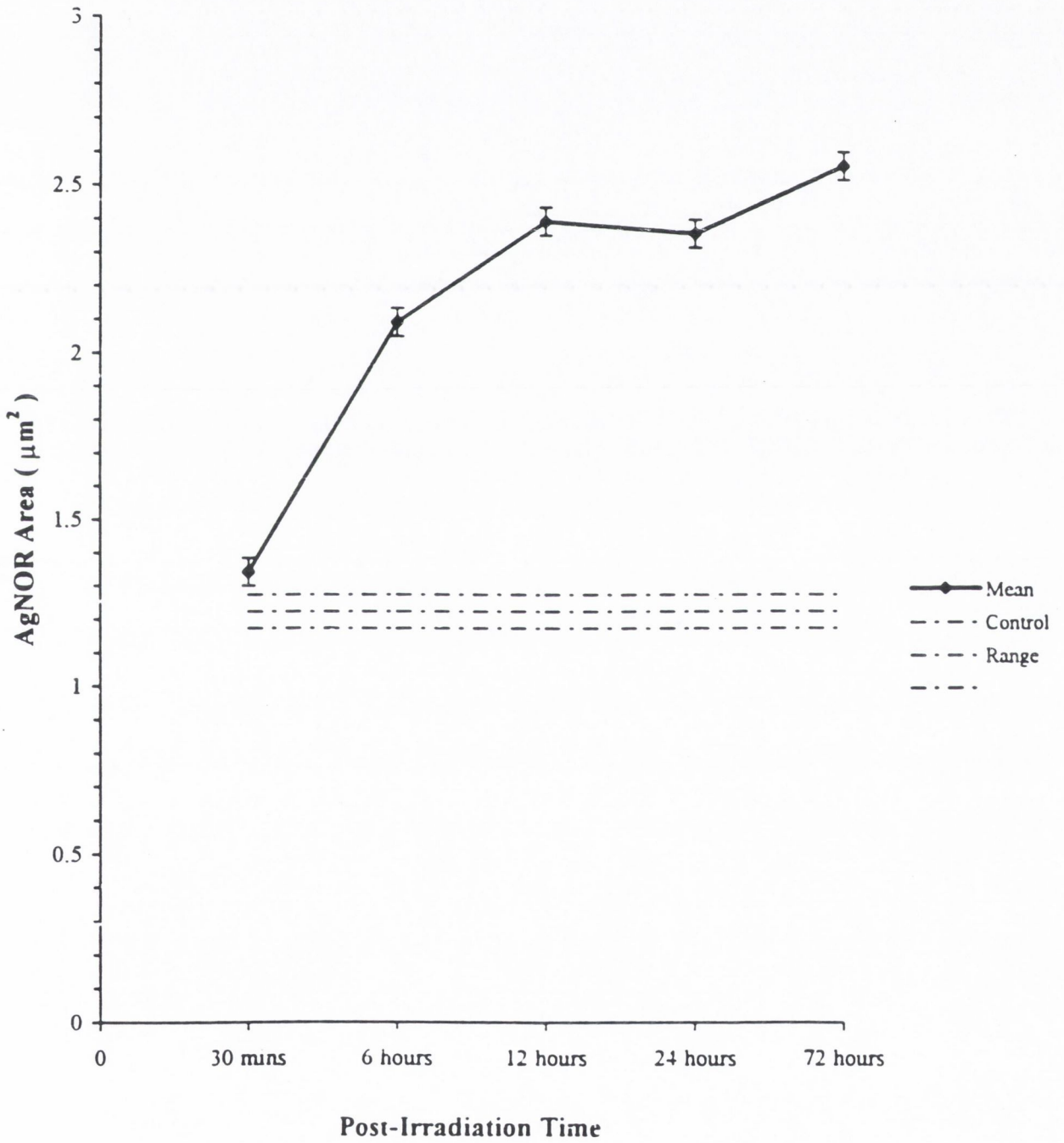
10Gy



Graph 11

AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

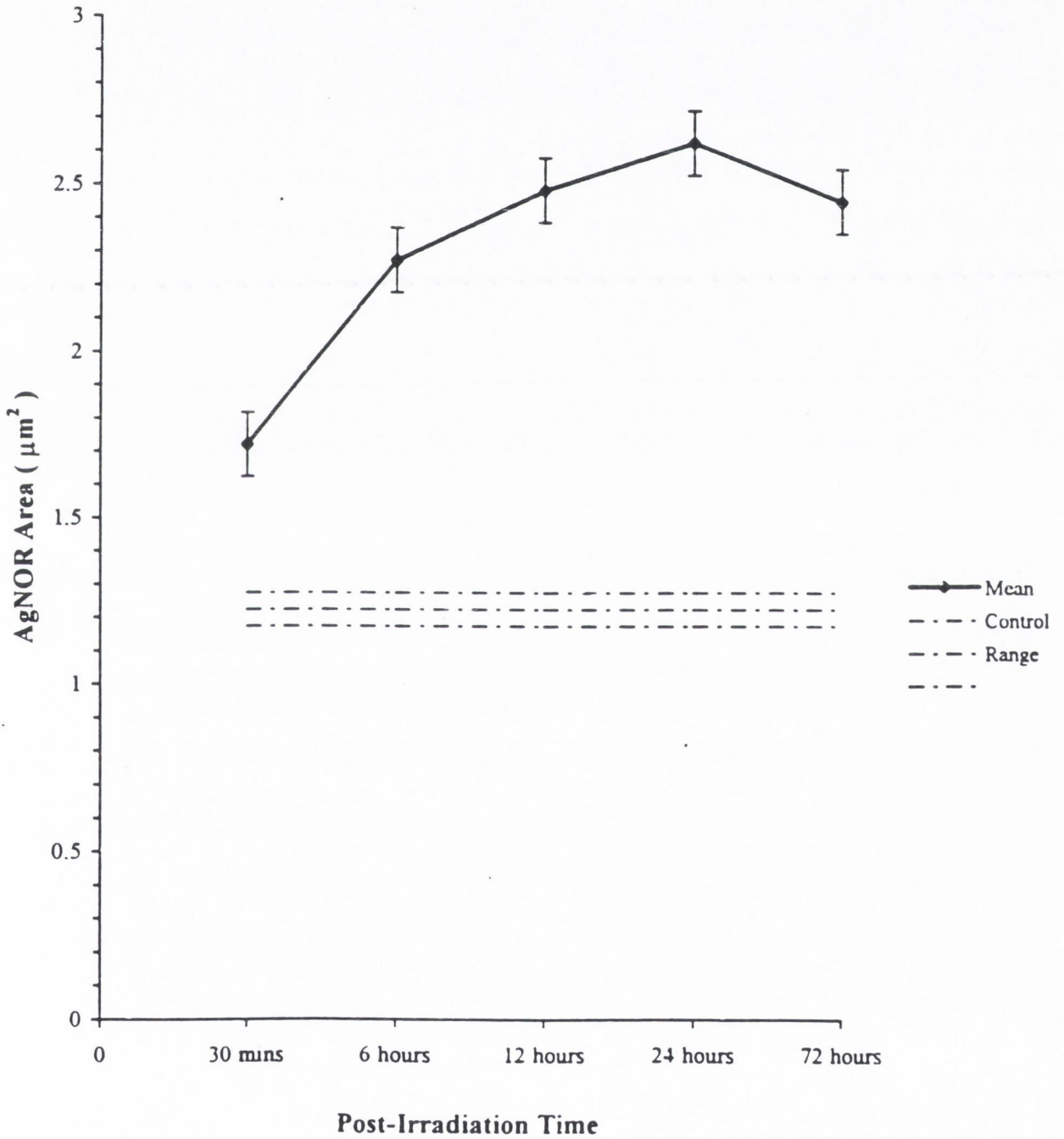
15Gy



Graph 12

AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

20Gy

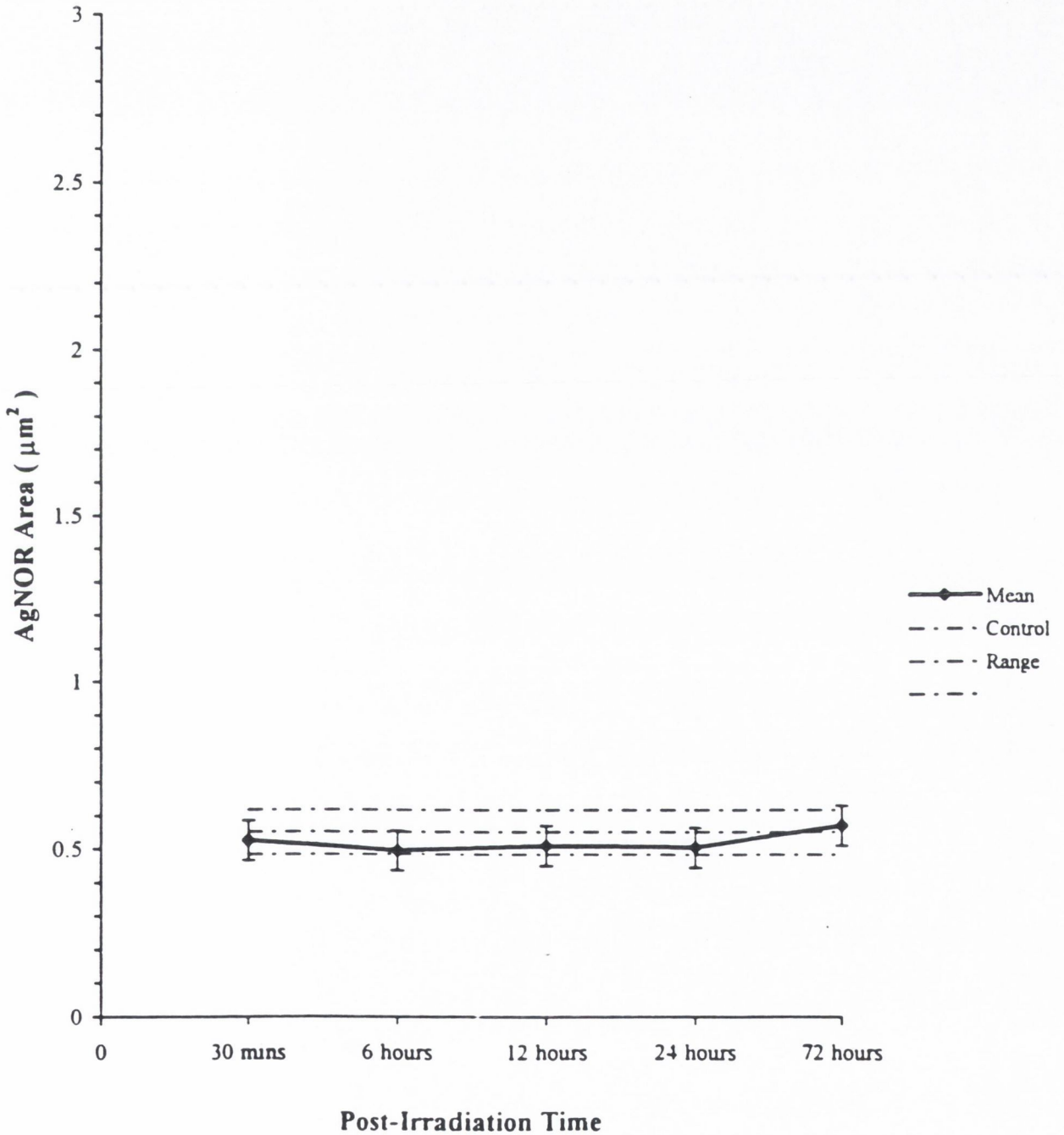


Graph 13

AgNOR Area vs Post-Irradiation Time

Villus Lamina Propria

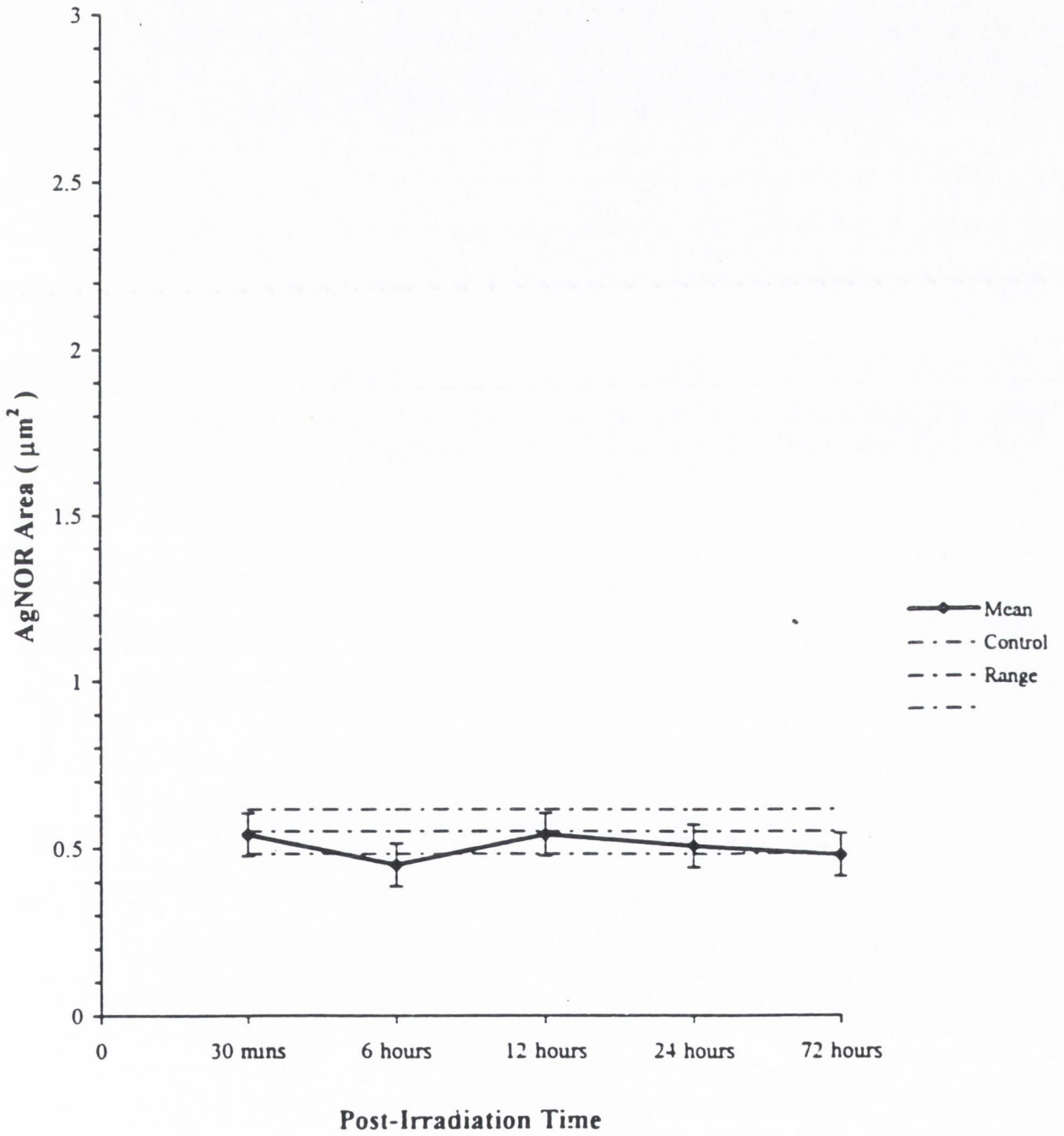
5Gy



Graph 14

AgNOR Area vs Post-Irradiation Time *Villus Lamina Propria*

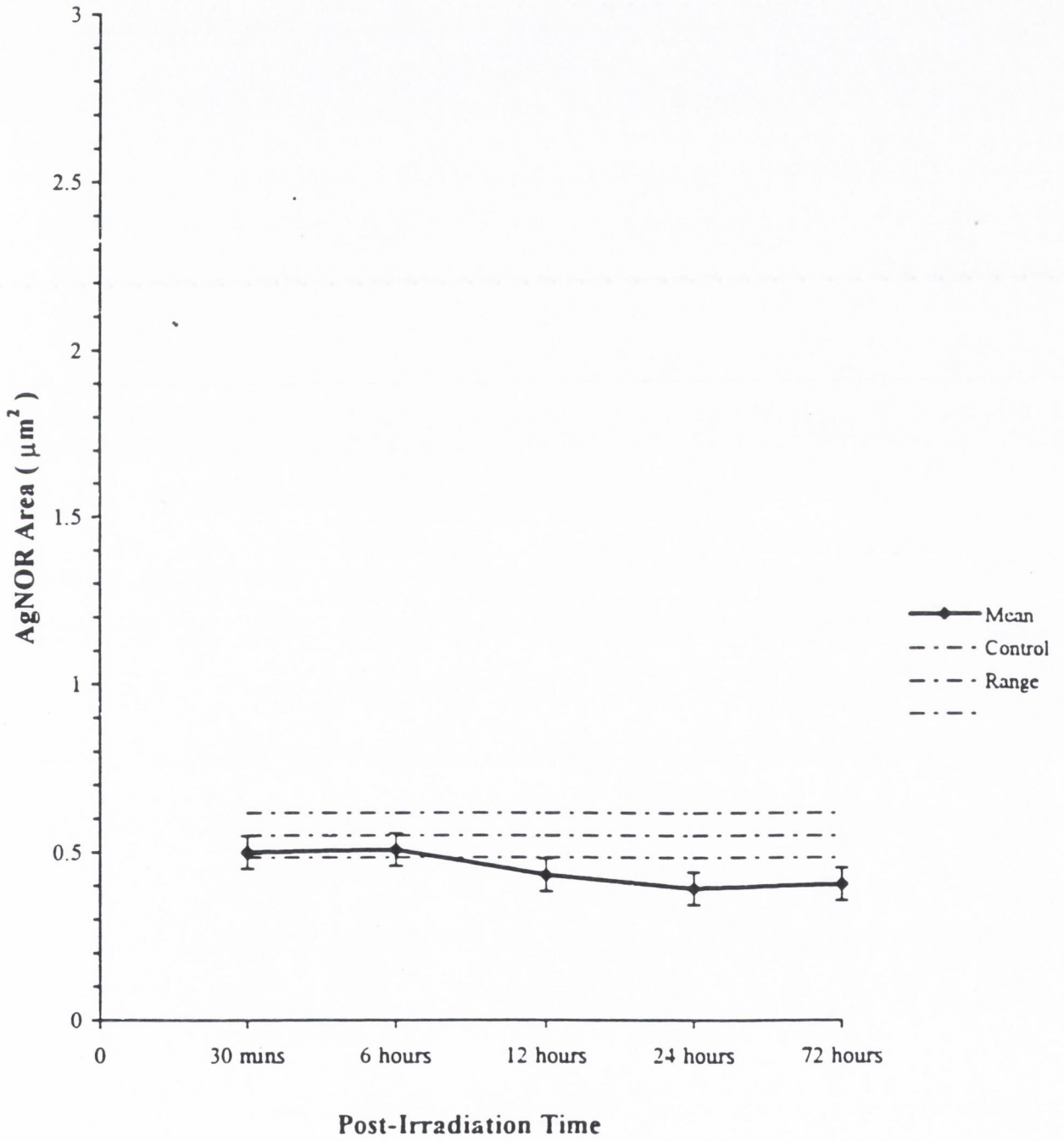
10Gy



Graph 15

AgNOR Area vs Post-Irradiation Time *Villus Lamina Propria*

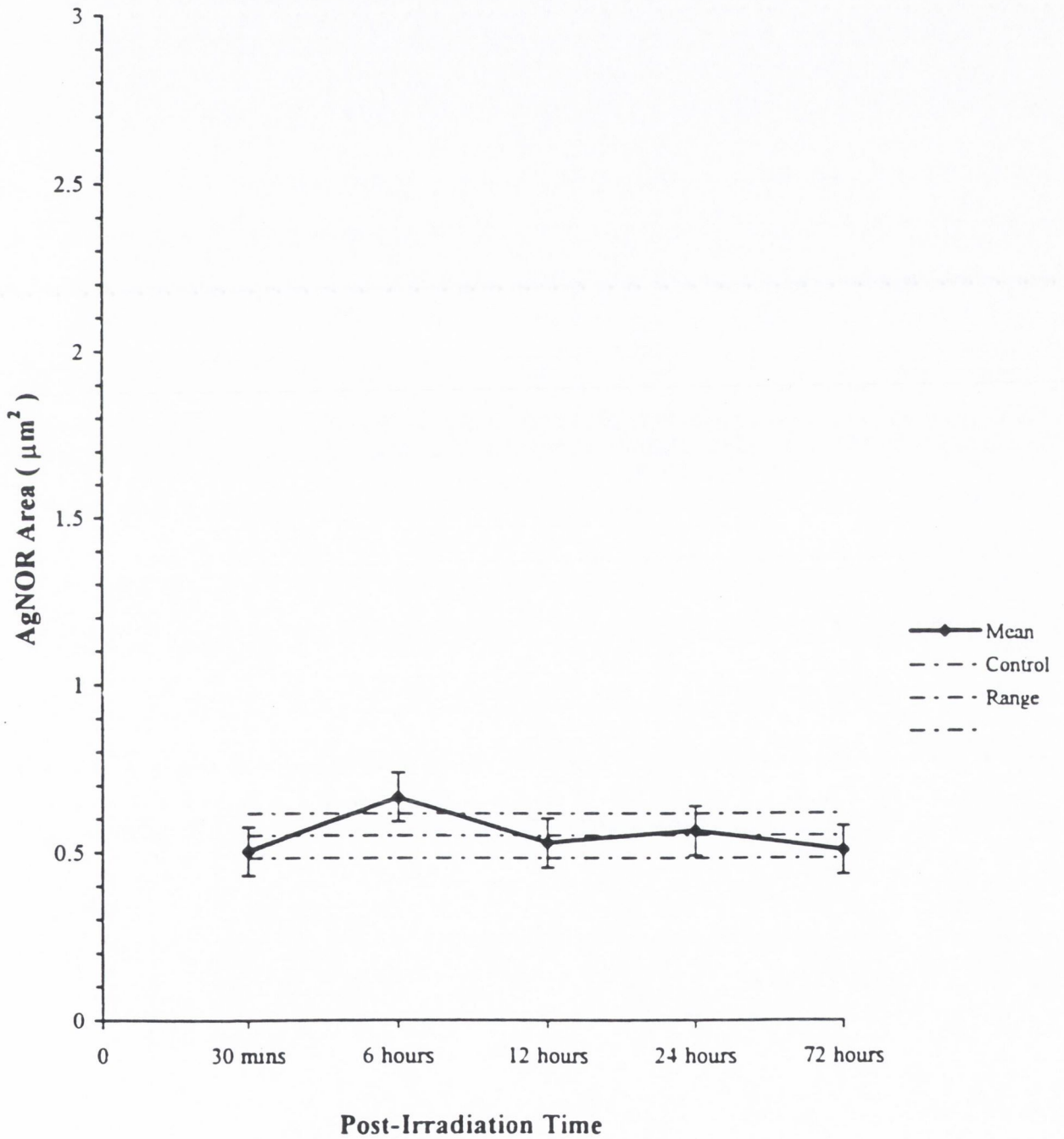
15Gy



Graph 16

AgNOR Area vs Post-Irradiation Time *Villus Lamina Propria*

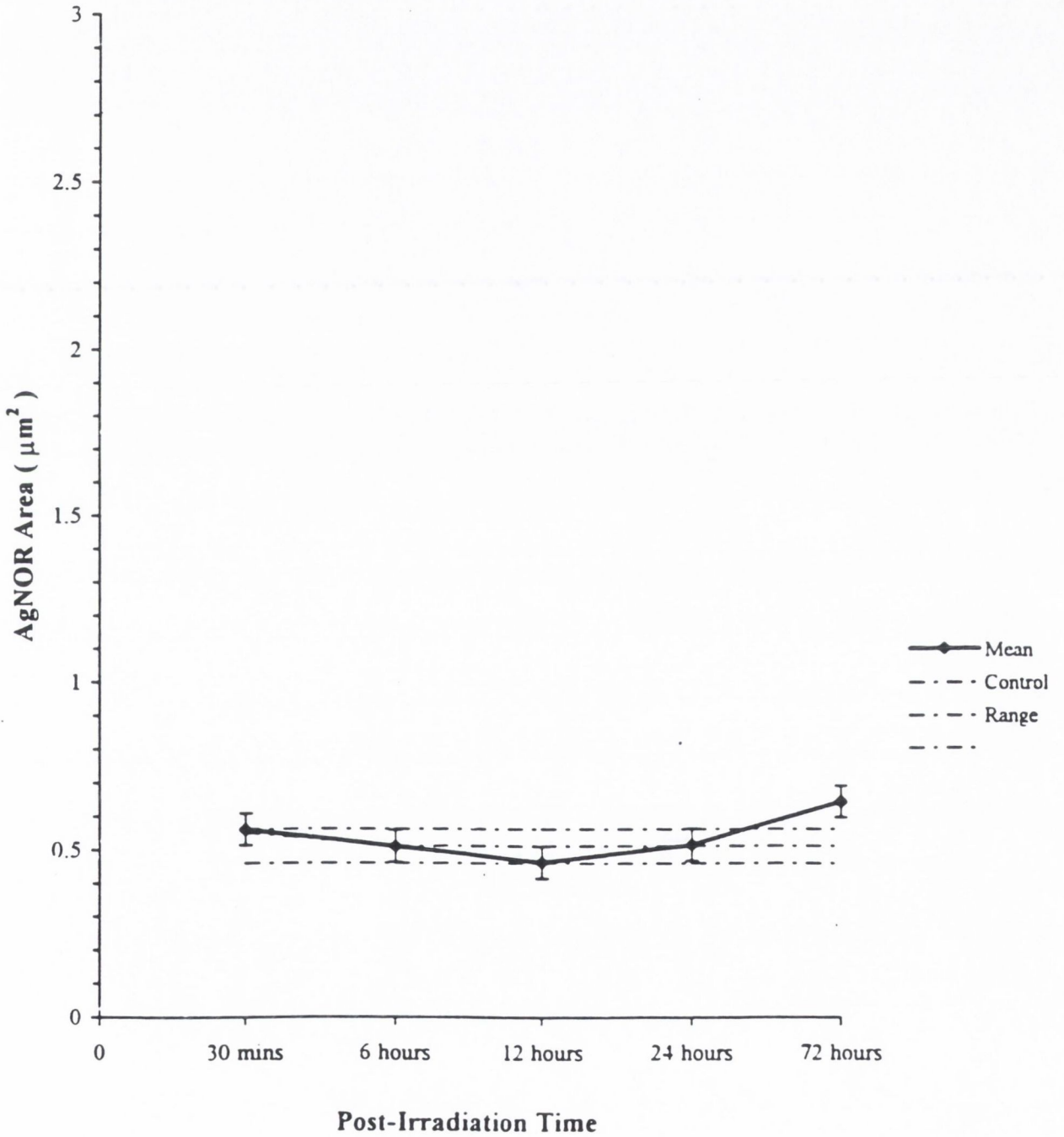
20Gy



Graph 17

AgNOR Area vs Post-Irradiation Time *Lamina Propria*

5Gy

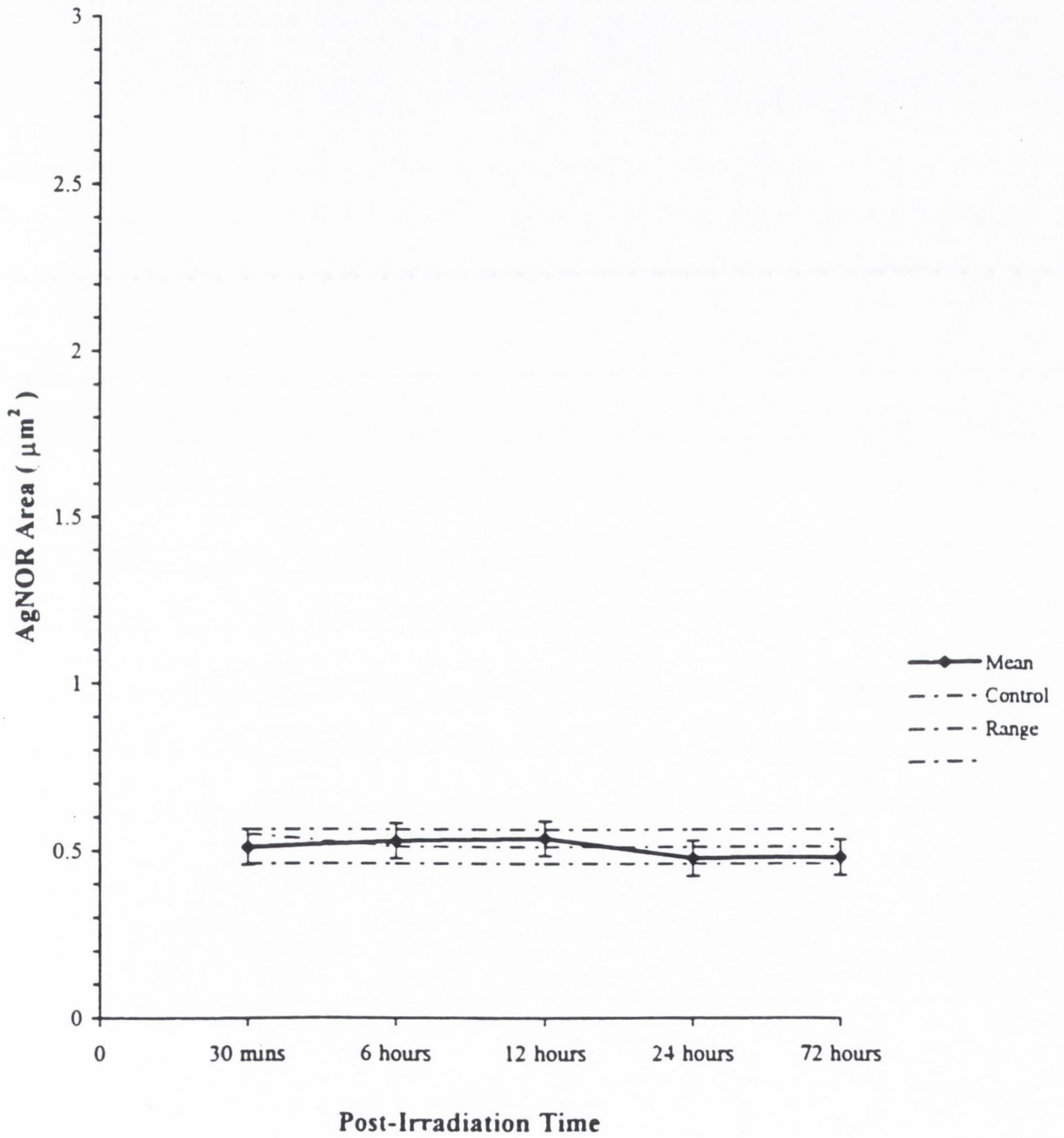


Graph 18

AgNOR Area vs Post-Irradiation Time

Lamina Propria

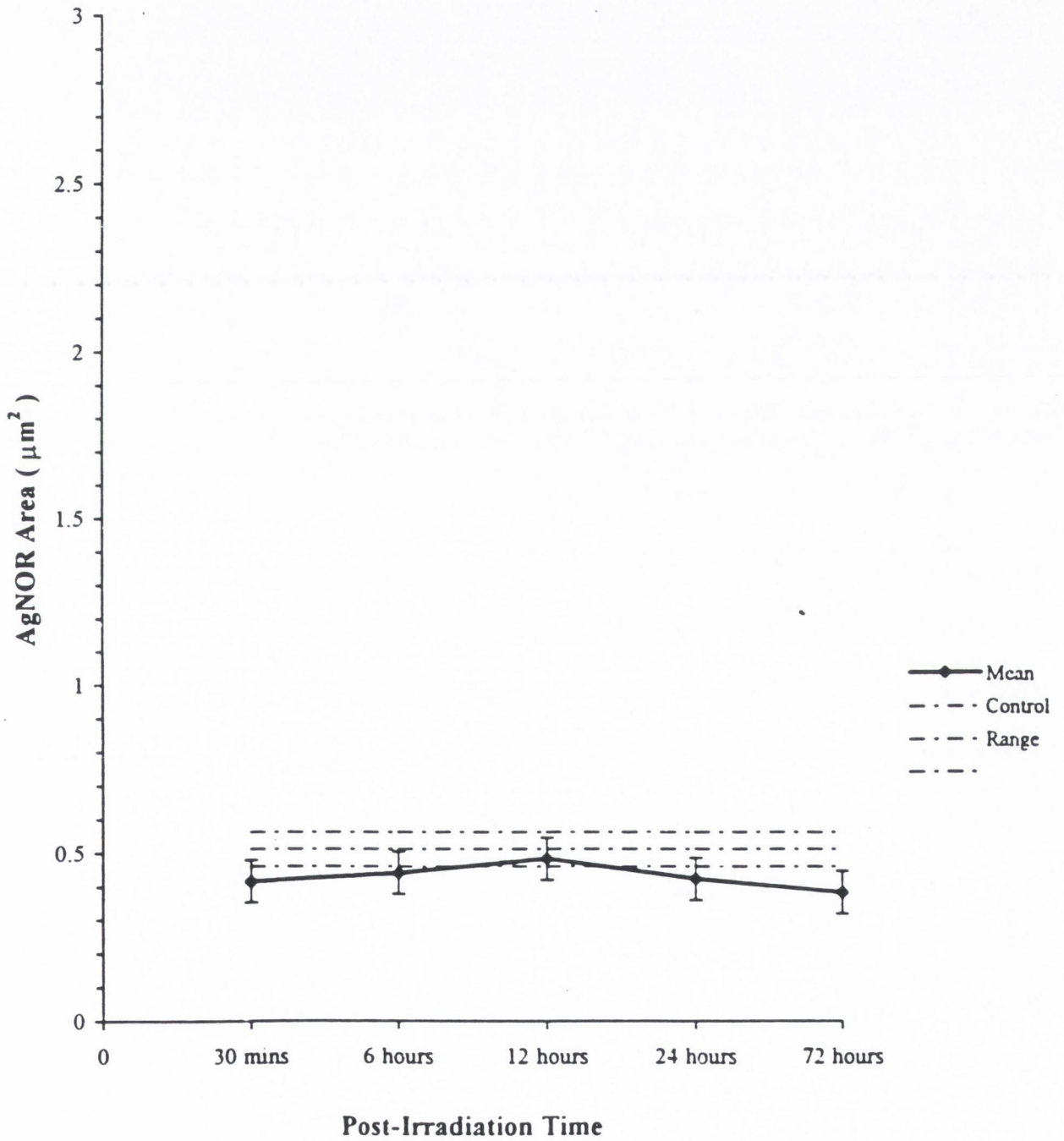
10Gy



Graph 19

AgNOR Area vs Post-Irradiation Time *Lamina Propria*

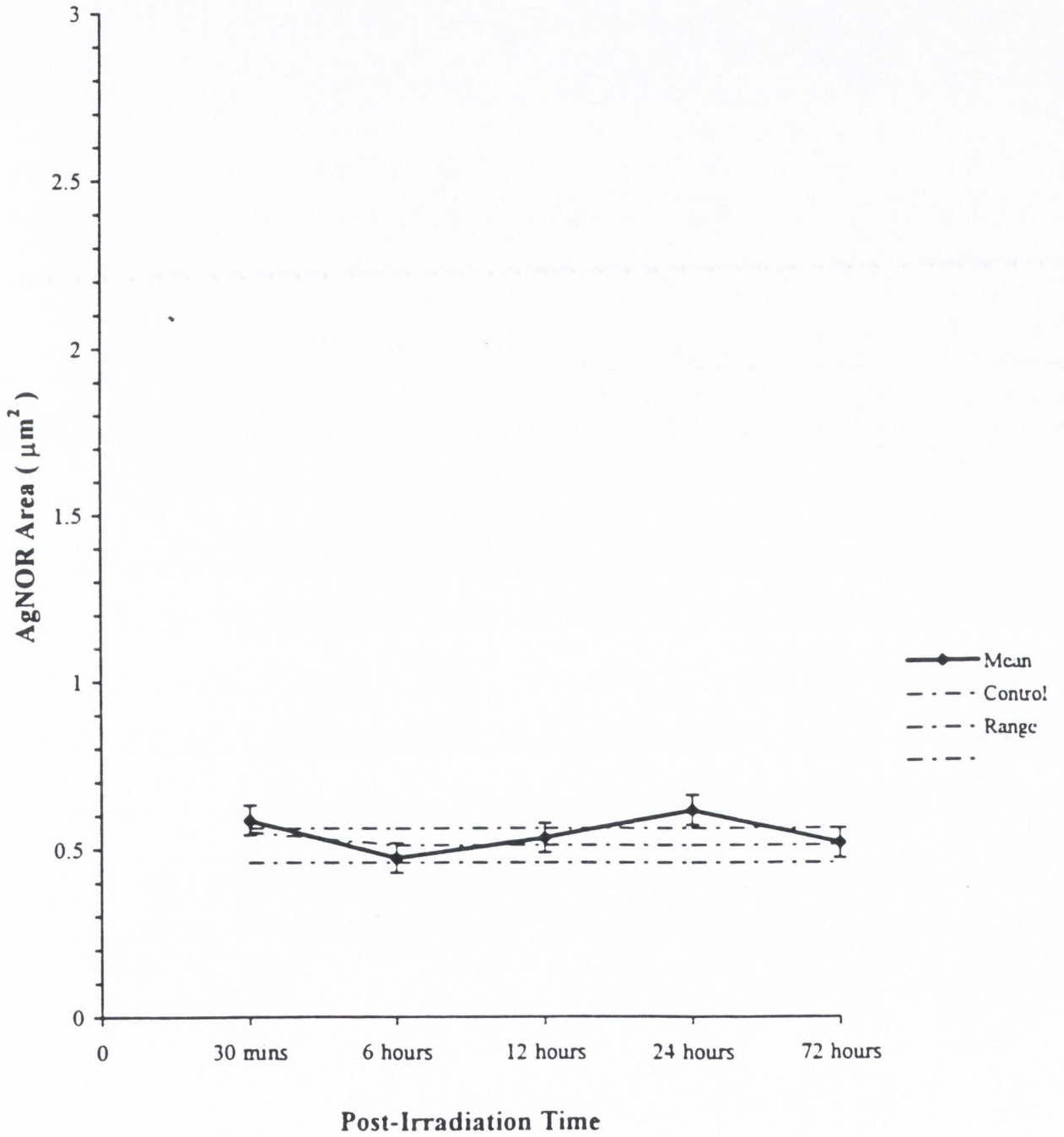
15Gy



Graph 20

AgNOR Area vs Post-Irradiation Time *Lamina Propria*

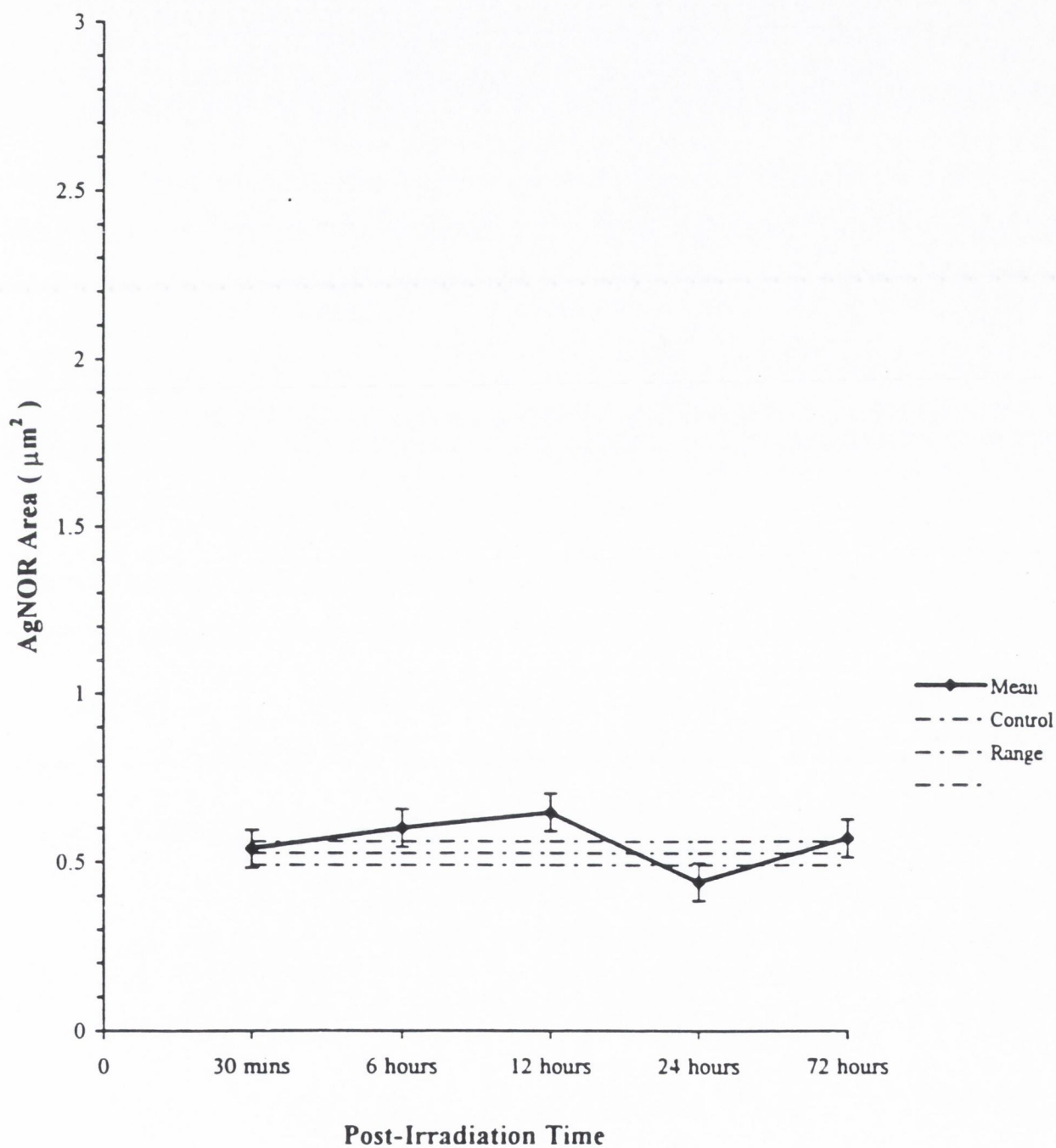
20Gy



Graph 21

AgNOR Area vs Post-Irradiation Time *Muscle Externa*

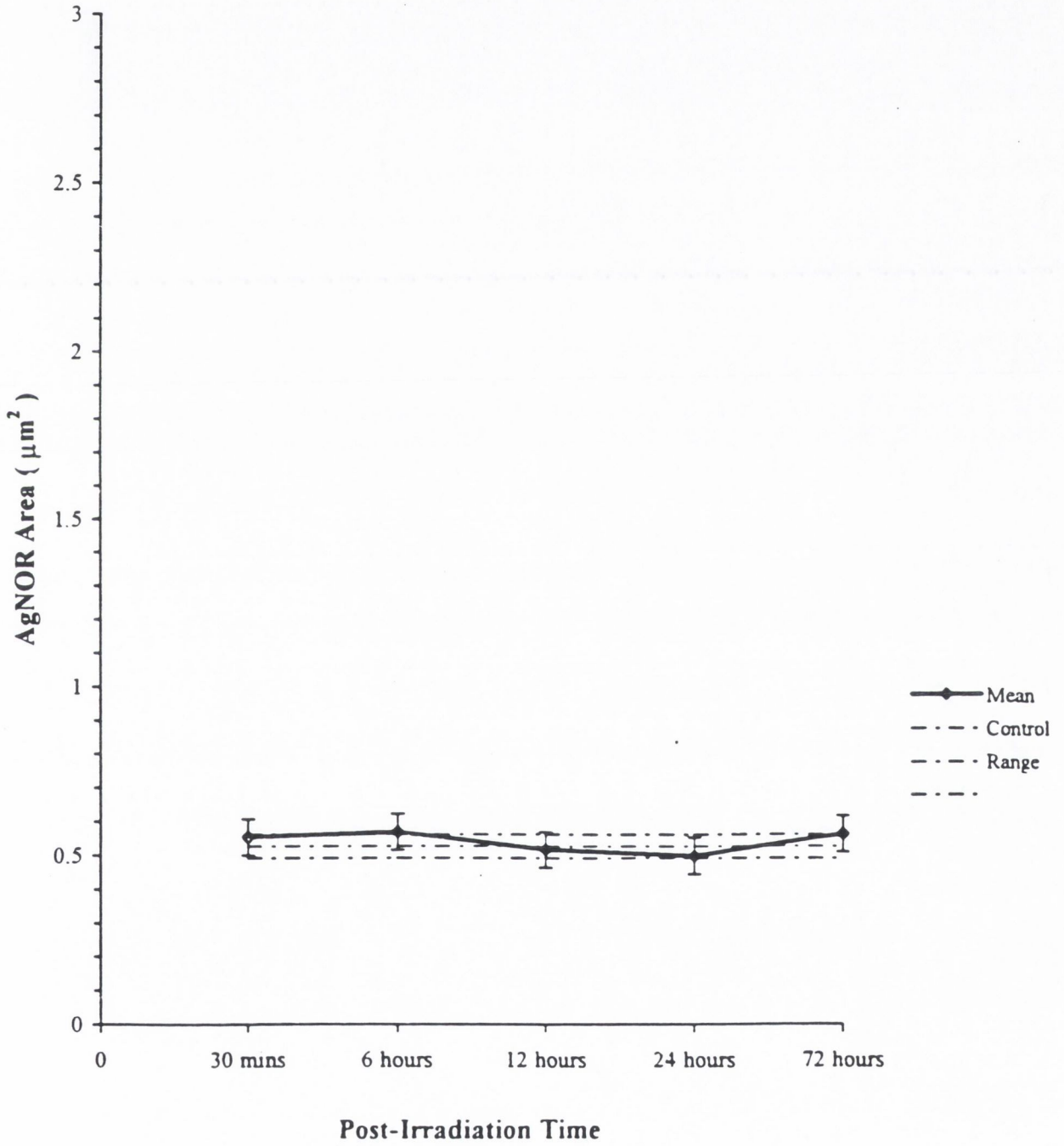
5Gy



Graph 22

AgNOR Area vs Post-Irradiation Time *Muscle Externa*

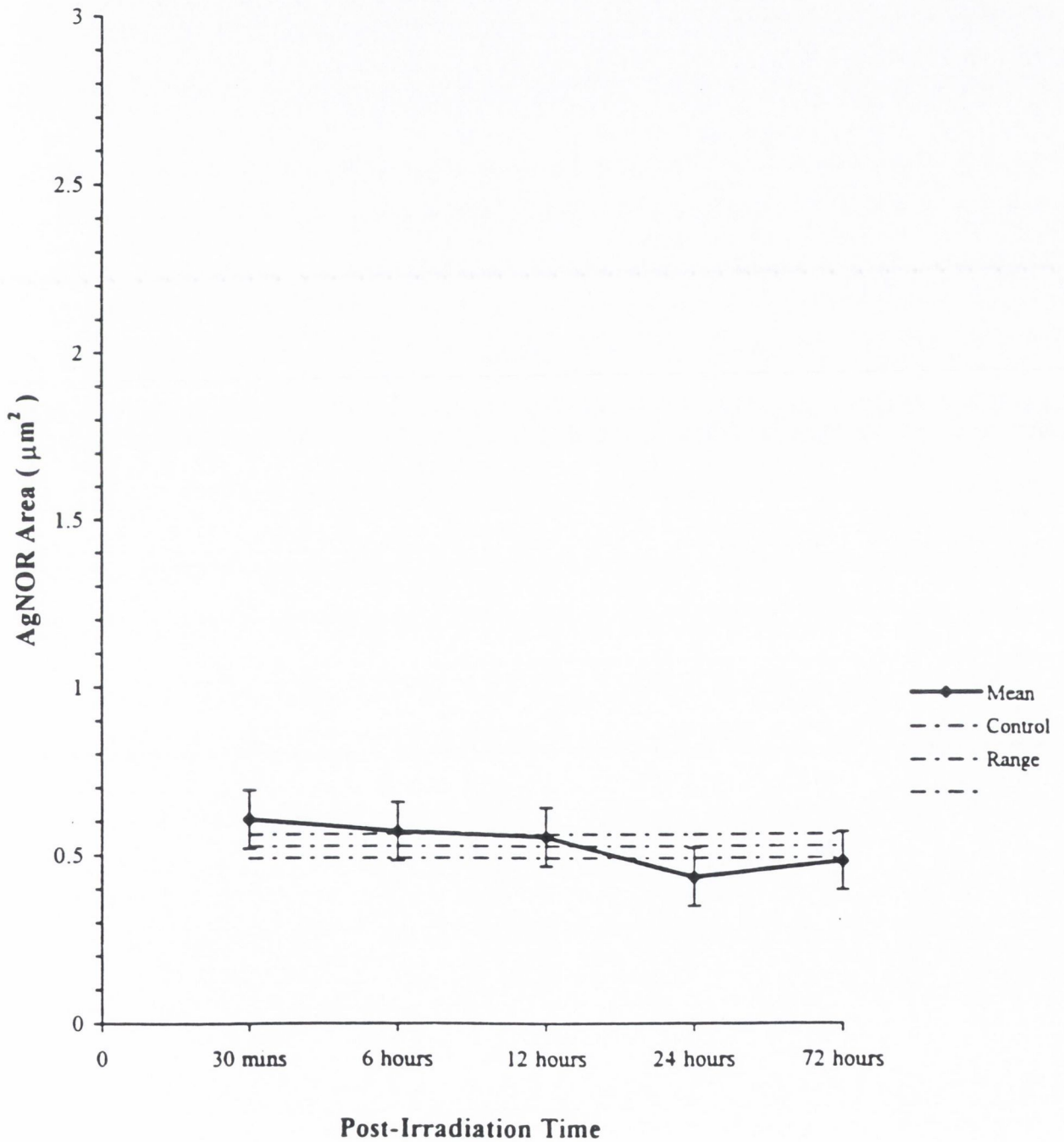
10Gy



Graph 23

AgNOR Area vs Post-Irradiation Time *Muscle Externa*

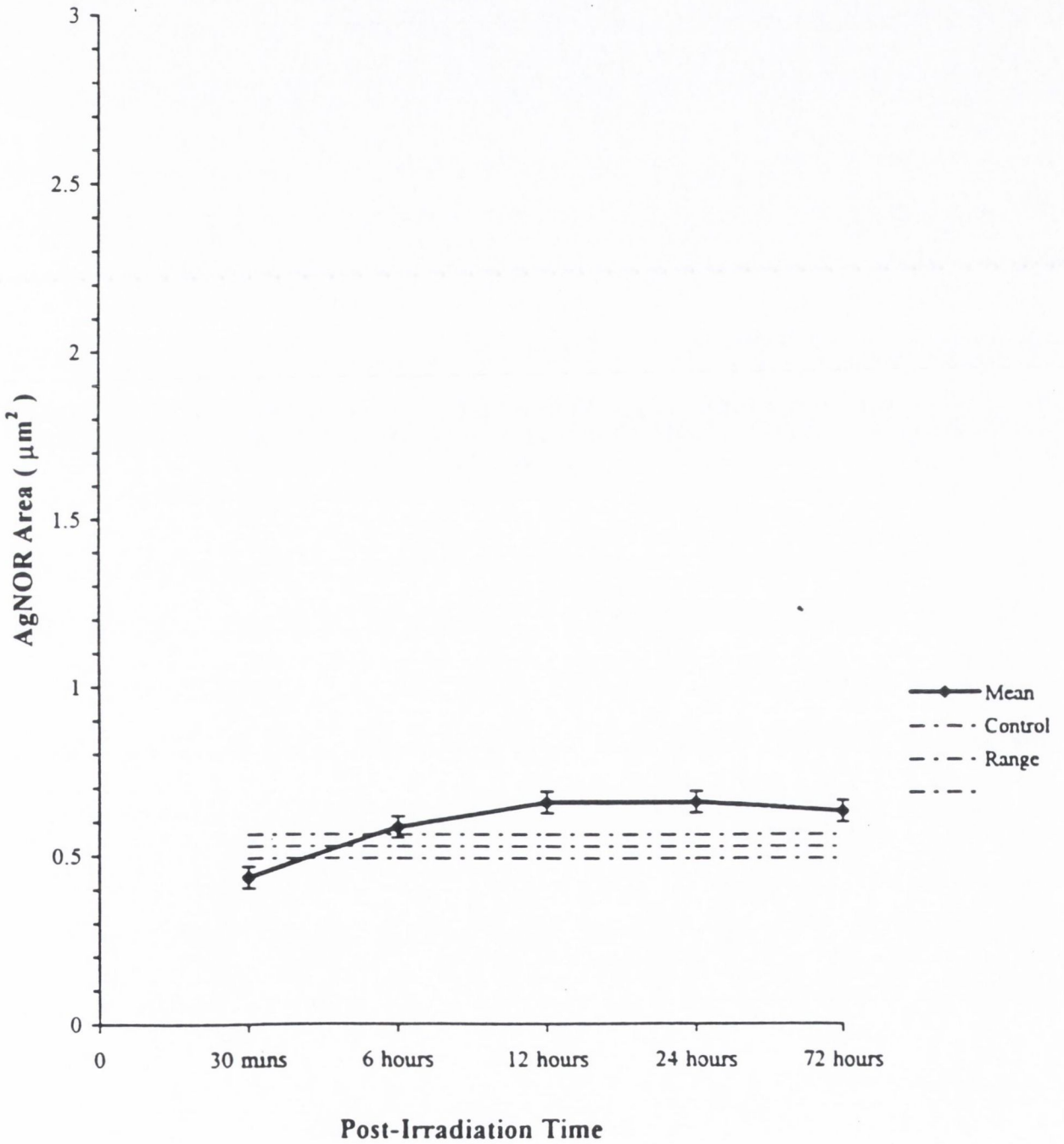
15Gy



Graph 24

AgNOR Area vs Post-Irradiation Time *Muscle Externa*

20Gy

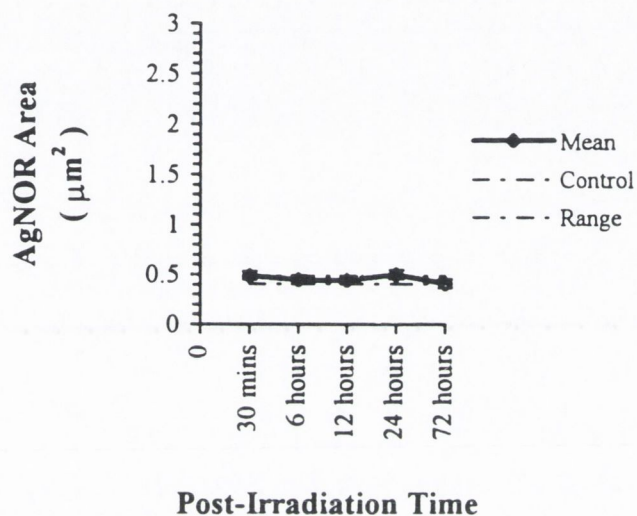


Graph 25

AgNOR Area vs Post-Irradiation Time

Upper Villus

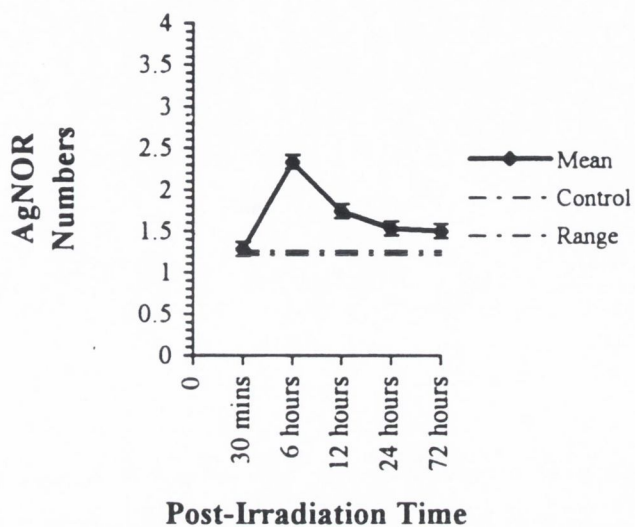
5Gy



AgNOR Numbers vs Post-Irradiation Time

Upper Villus

5Gy

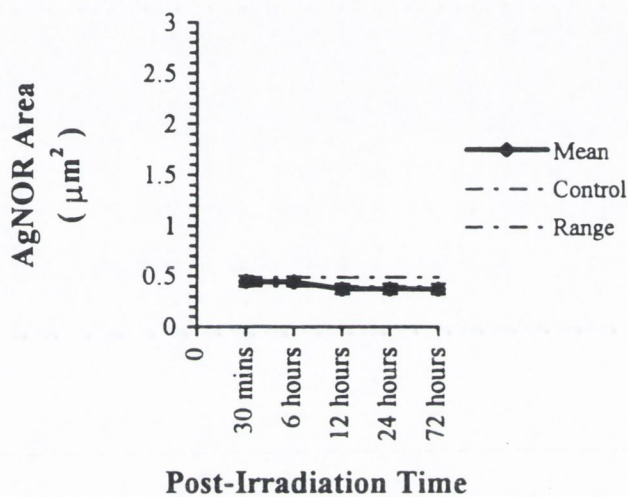


Graph 26

AgNOR Area vs Post-Irradiation Time

Upper Villus

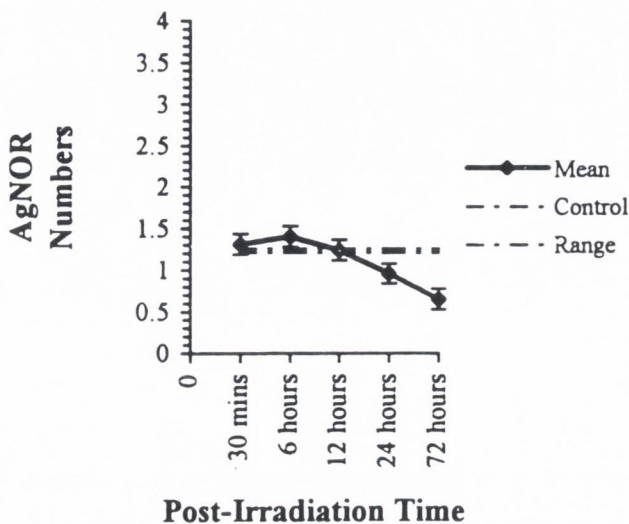
10Gy



AgNOR Numbers vs Post-Irradiation Time

Upper Villus

10Gy

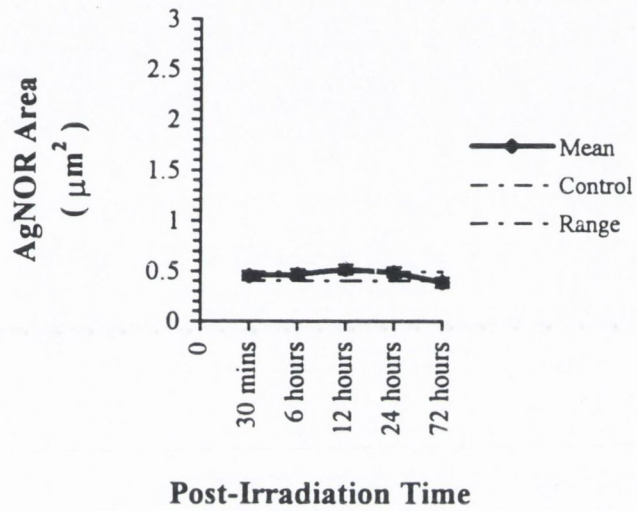


Graph 27

AgNOR Area vs Post-Irradiation Time

Upper Villus

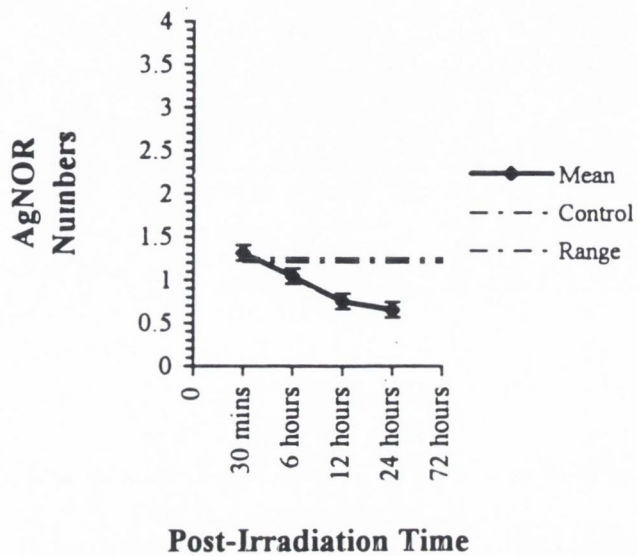
15Gy



AgNOR Numbers vs Post-Irradiation Time

Upper Villus

15Gy

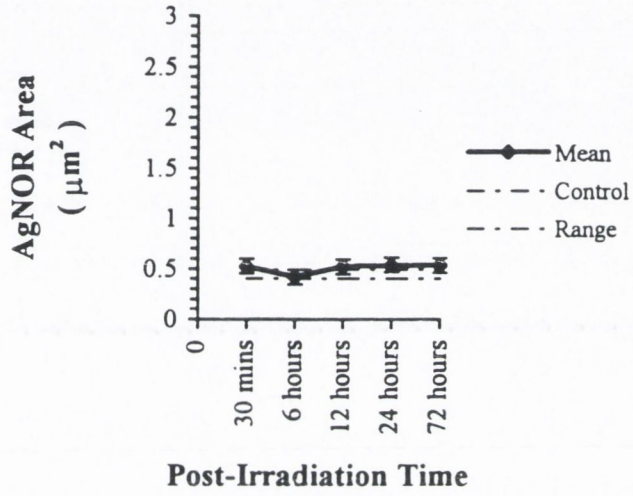


Graph 28

AgNOR Area vs Post-Irradiation Time

Upper Villus

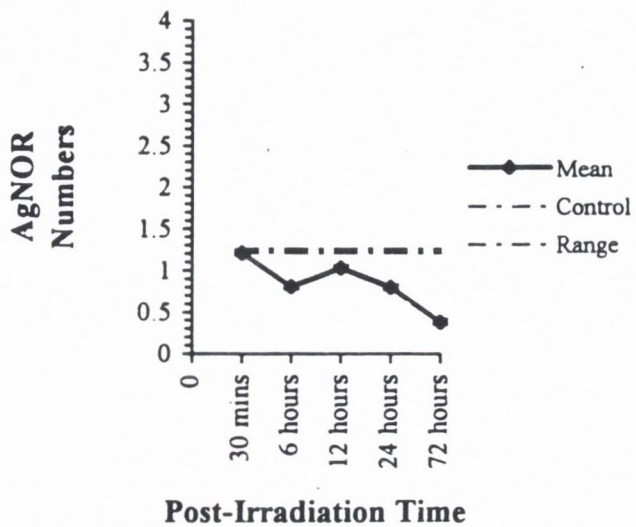
20Gy



AgNOR Numbers vs Post-Irradiation Time

Upper Villus

20Gy

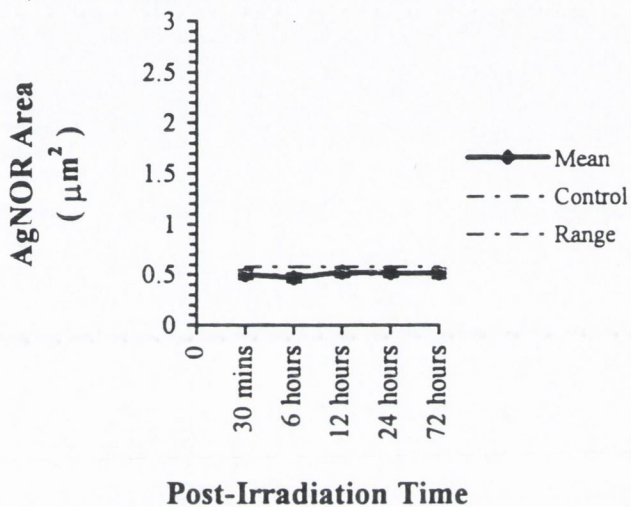


Graph 29

AgNOR Area vs Post-Irradiation Time

Lower Villus

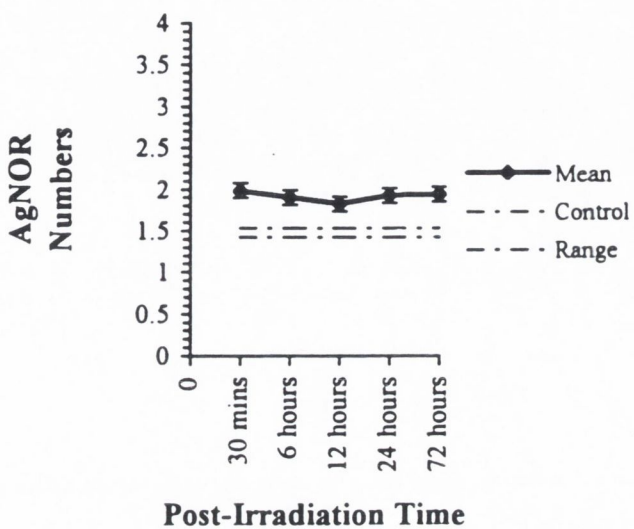
5Gy



AgNOR Numbers vs Post-Irradiation Time

Lower Villus

5Gy

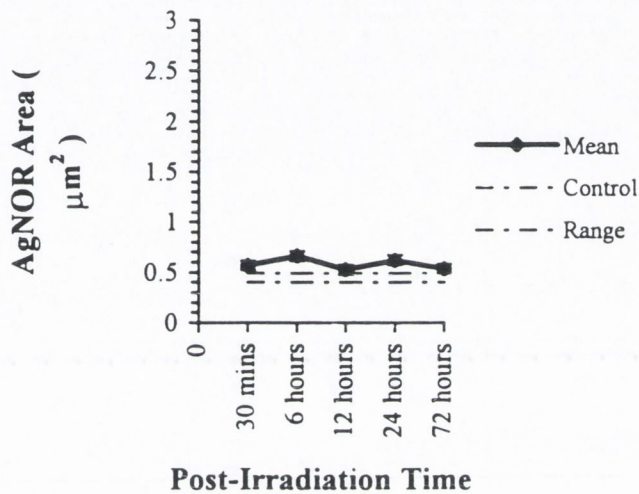


Graph 30

AgNOR Area vs Post-Irradiation Time

Lower Villus

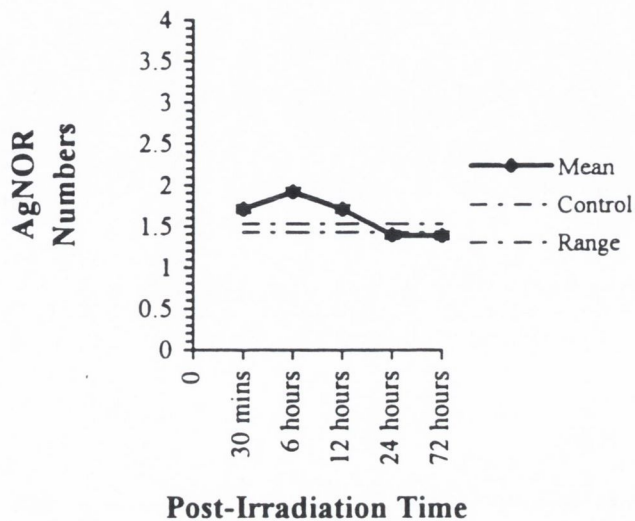
10Gy



AgNOR Numbers vs Post-Irradiation Time

Lower Villus

10Gy

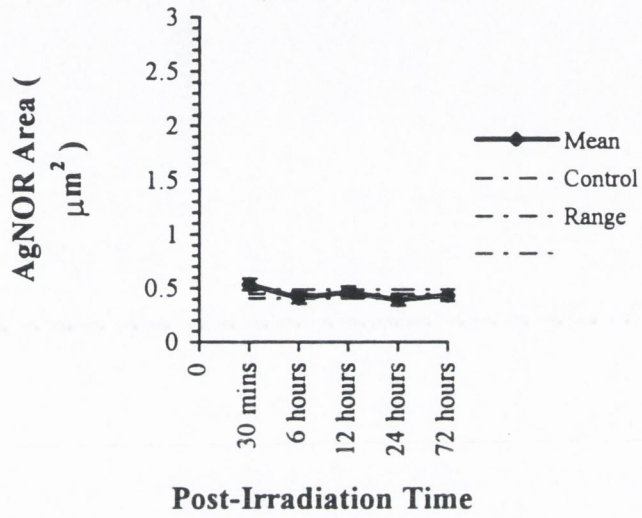


Graph 31

AgNOR Area vs Post-Irradiation Time

Lower Villus

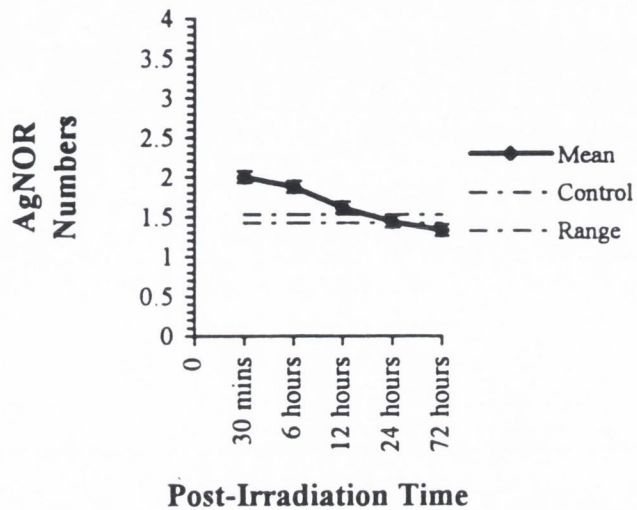
15Gy



AgNOR Numbers vs Post-Irradiation Time

Lower Villus

15Gy

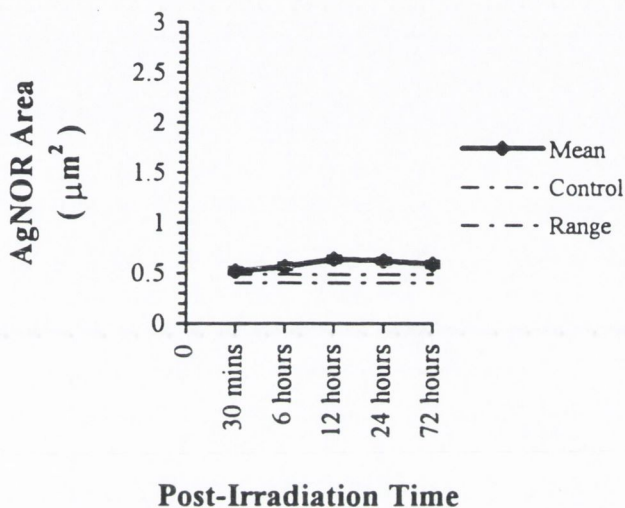


Graph 32

AgNOR Area vs Post-Irradiation Time

Lower Villus

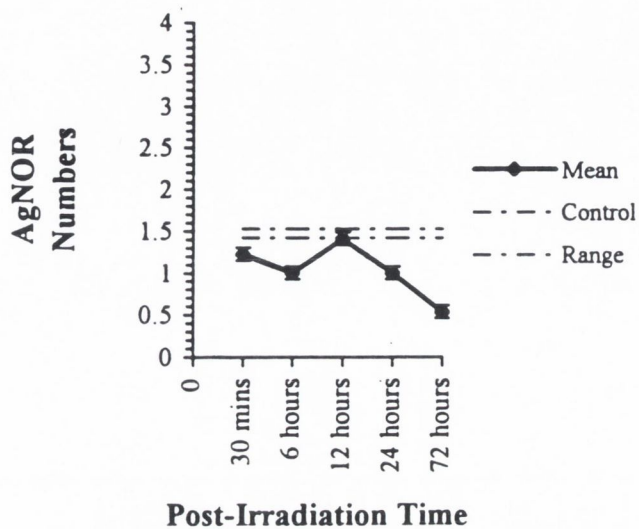
20Gy



AgNOR Numbers vs Post-Irradiation Time

Lower Villus

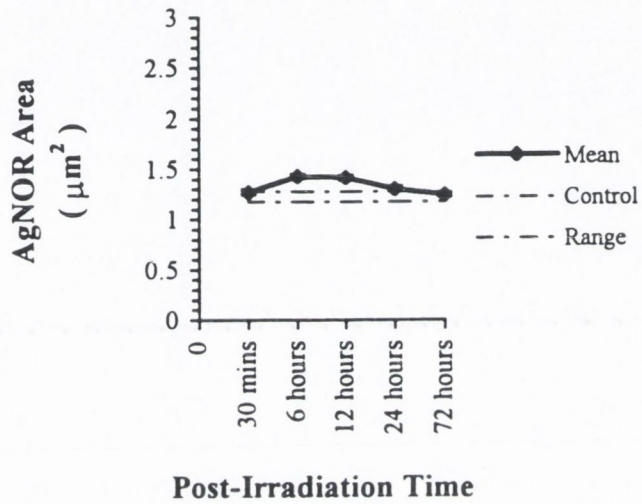
20Gy



Graph 33

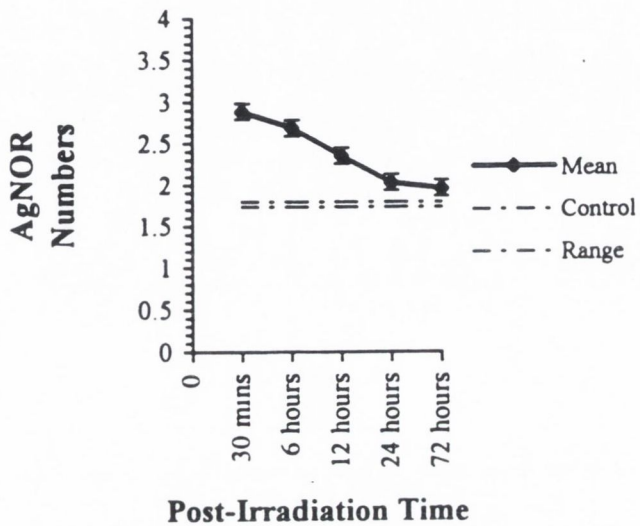
AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

5Gy



AgNOR Numbers vs Post-Irradiation Time *Cryptal Epithelium*

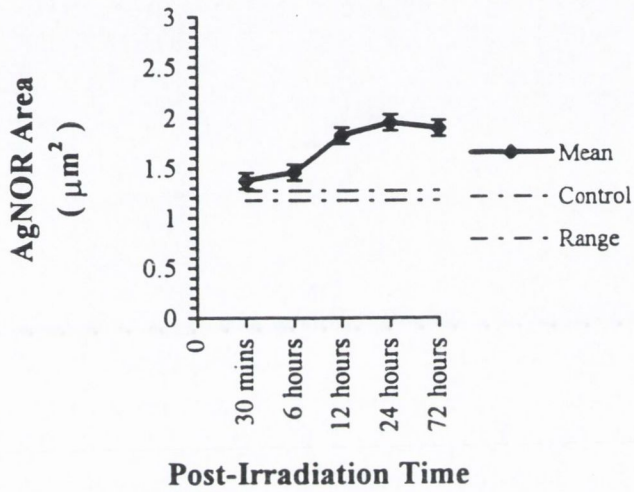
5Gy



Graph 34

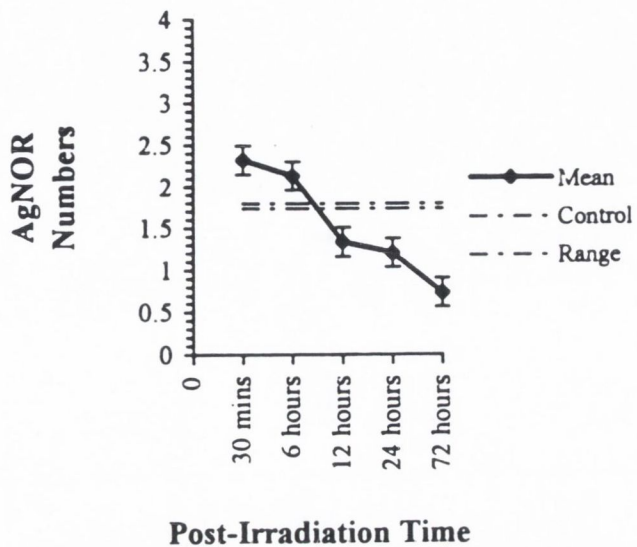
AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

10Gy



AgNOR Numbers vs Post-Irradiation Time *Cryptal Epithelium*

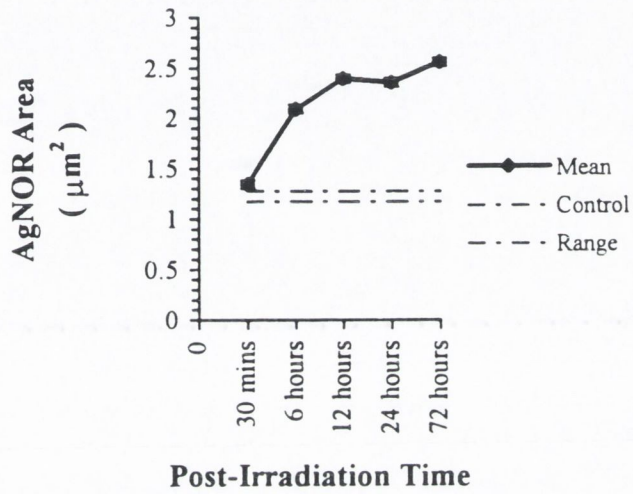
10Gy



Graph 35

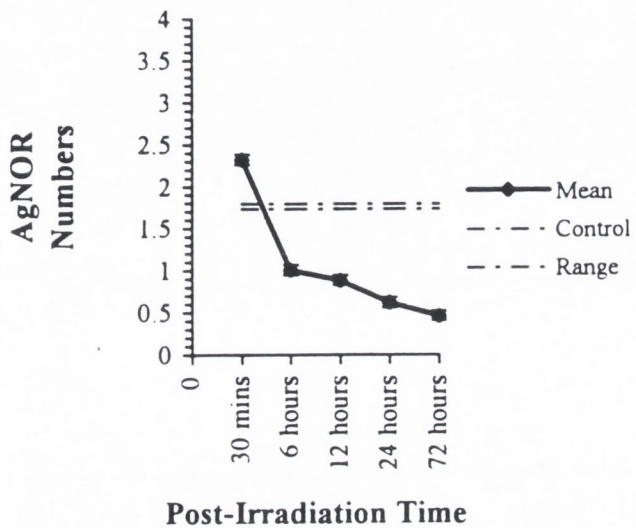
AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

15Gy



AgNOR Numbers vs Post-Irradiation Time *Cryptal Epithelium*

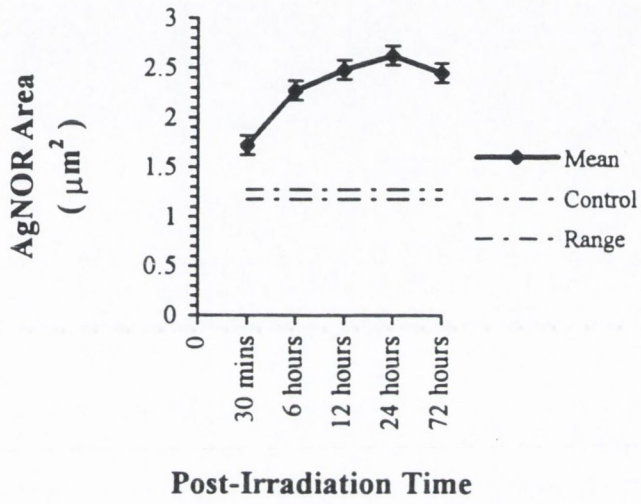
15Gy



Graph 36

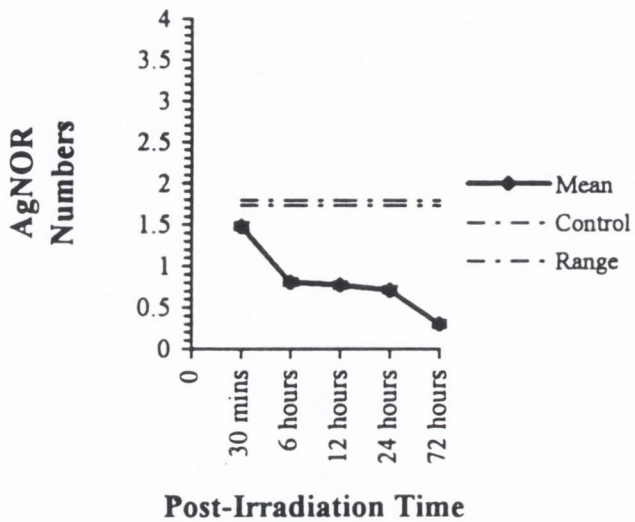
AgNOR Area vs Post-Irradiation Time *Cryptal Epithelium*

20Gy



AgNOR Numbers vs Post-Irradiation Time *Cryptal Epithelium*

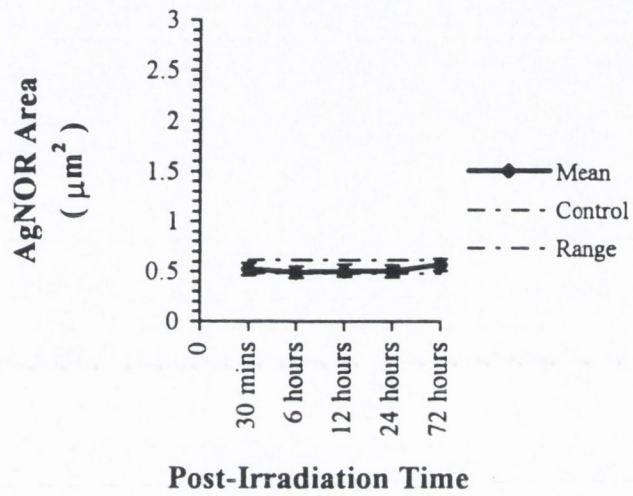
20Gy



Graph 37

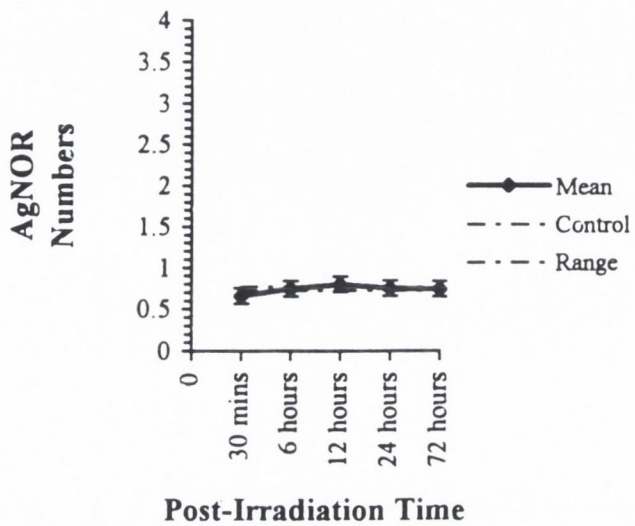
AgNOR Area vs Post-Irradiation Time *Villus Lamina Propria*

5Gy



AgNOR Numbers vs Post-Irradiation Time *Villus Lamina Propria*

5Gy

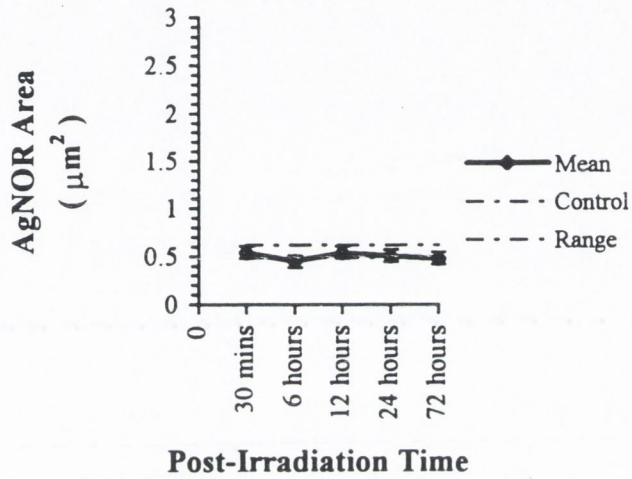


Graph 38

AgNOR Area vs Post-Irradiation Time

Villus Lamina Propria

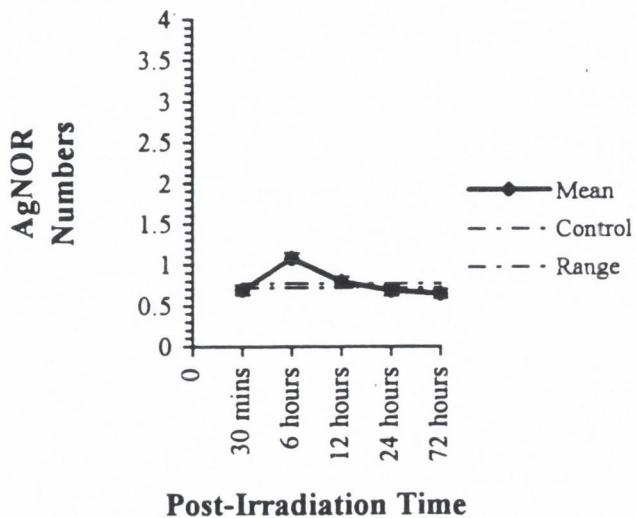
10Gy



AgNOR Numbers vs Post-Irradiation Time

Villus Lamina Propria

10Gy

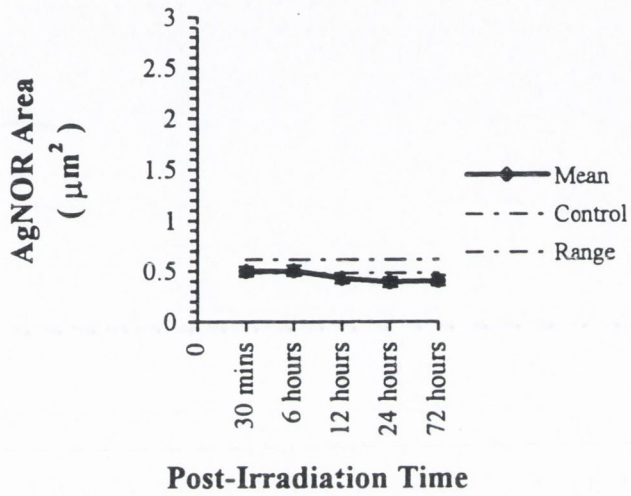


Graph 39

AgNOR Area vs Post-Irradiation Time

Villus Lamina Propria

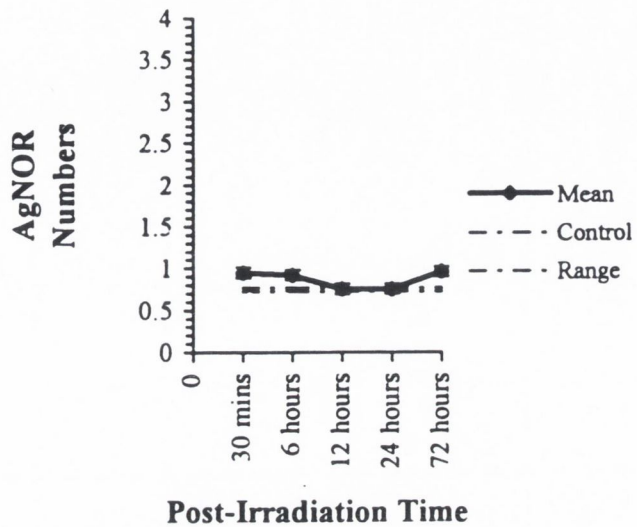
15Gy



AgNOR Numbers vs Post-Irradiation Time

Villus Lamina Propria

15Gy

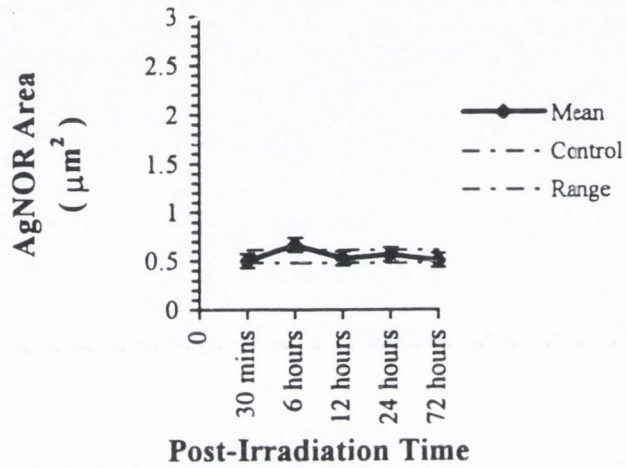


Graph 40

AgNOR Area vs Post-Irradiation Time

Villus Lamina Propria

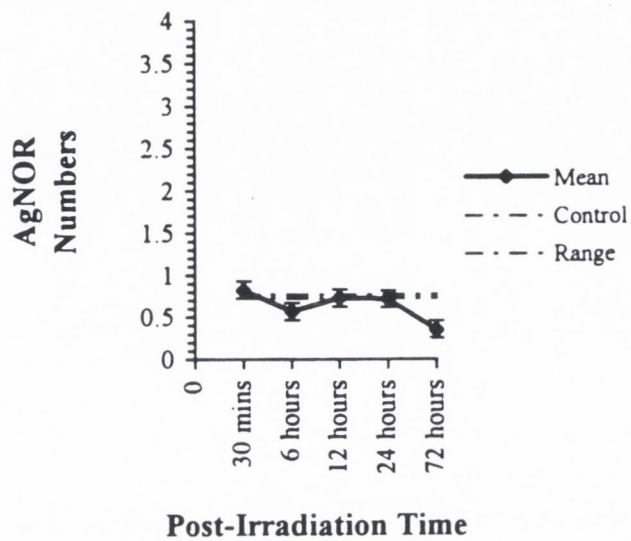
20Gy



AgNOR Numbers vs Post-Irradiation Time

Villus Lamina Propria

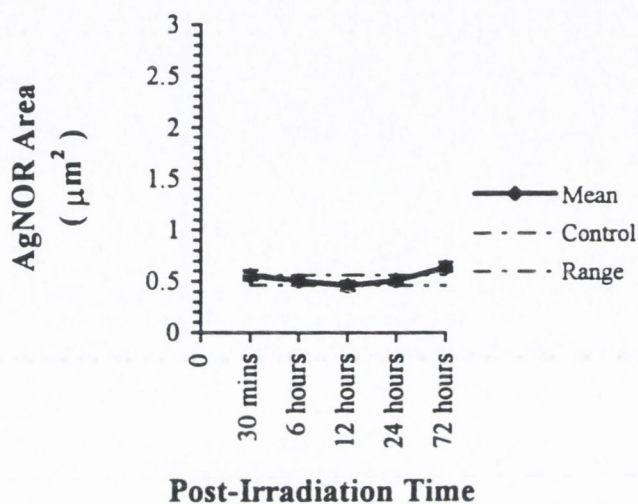
20Gy



Graph 41

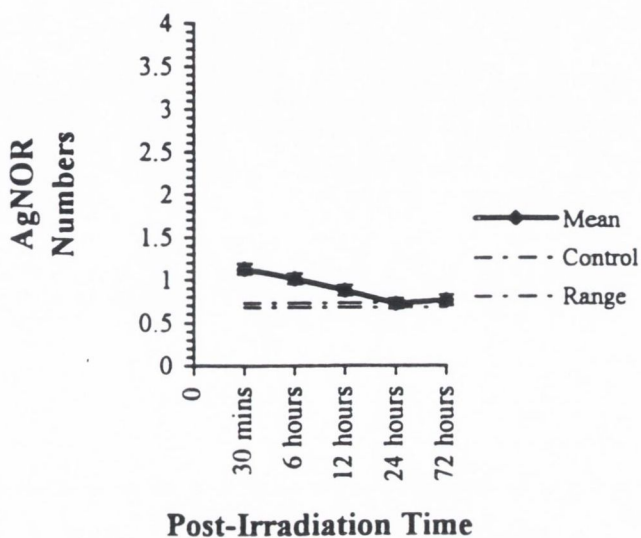
AgNOR Area vs Post-Irradiation Time *Lamina Propria*

5Gy



AgNOR Numbers vs Post-Irradiation Time *Lamina Propria*

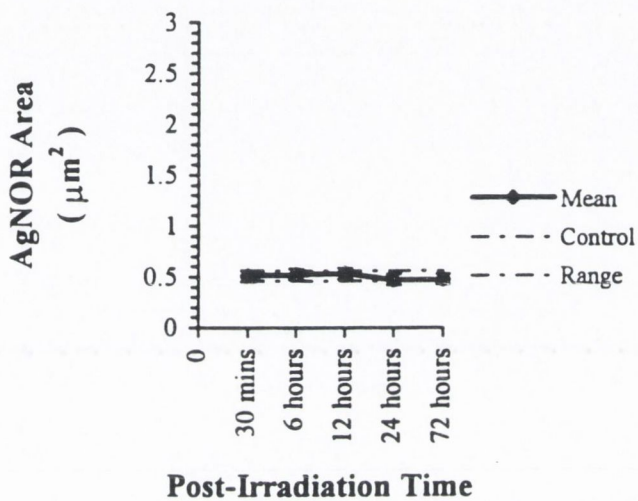
5Gy



Graph 42

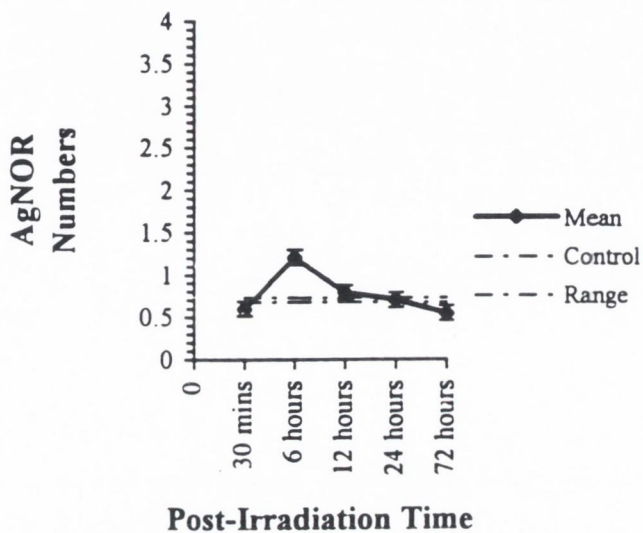
AgNOR Area vs Post-Irradiation Time
Lamina Propria

10Gy



AgNOR Numbers vs Post-Irradiation Time
Lamina Propria

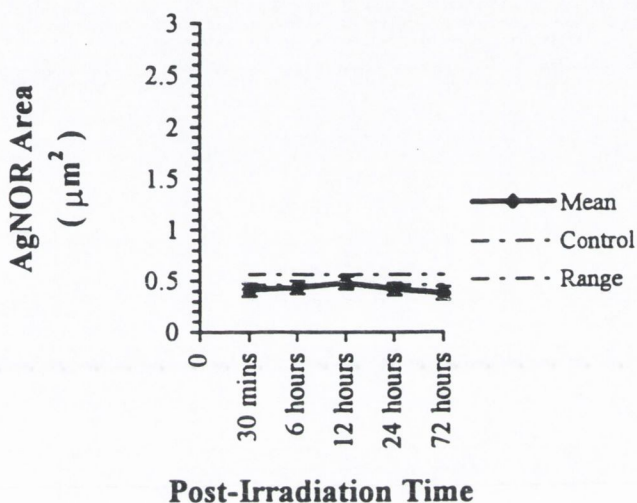
10Gy



Graph 43

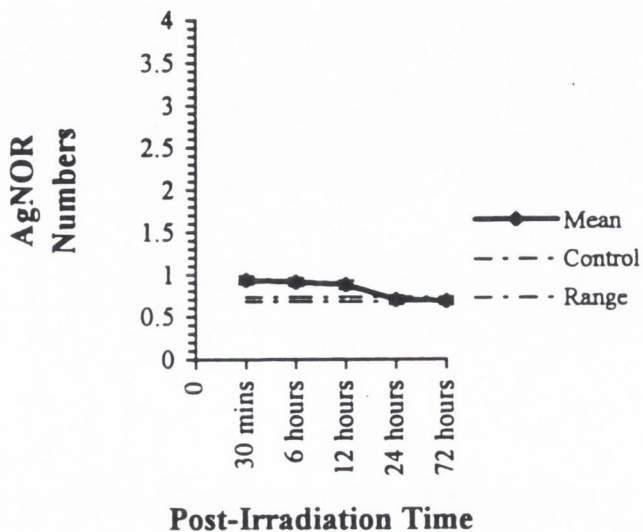
AgNOR Area vs Post-Irradiation Time *Lamina Propria*

15Gy



AgNOR Numbers vs Post-Irradiation Time *Lamina Propria*

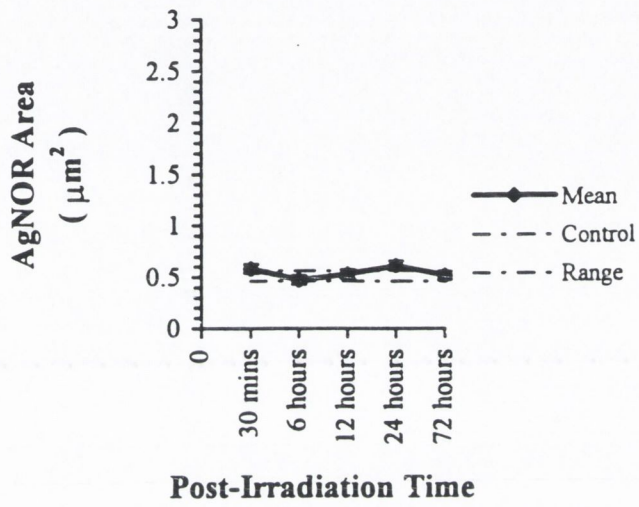
15Gy



Graph 44

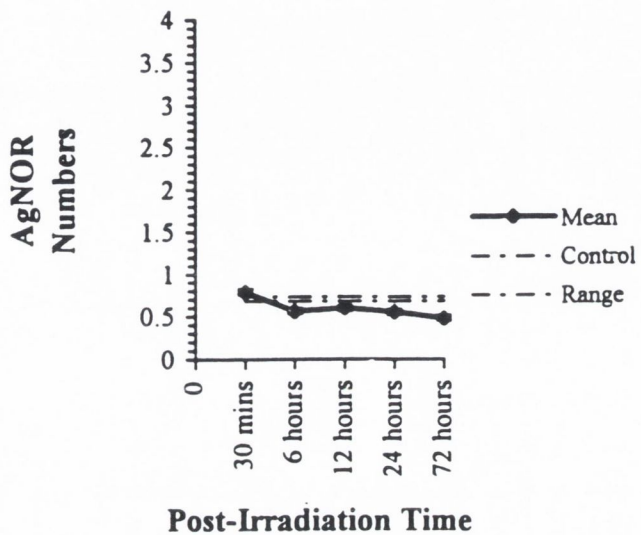
AgNOR Area vs Post-Irradiation Time *Lamina Propria*

20Gy



AgNOR Numbers vs Post-Irradiation Time *Lamina Propria*

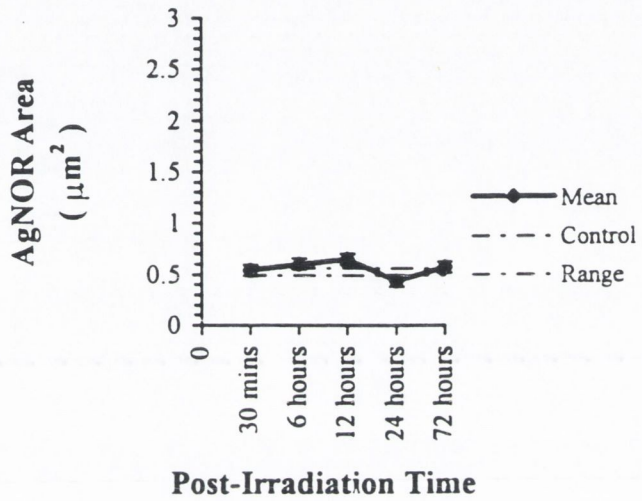
20Gy



Graph 45

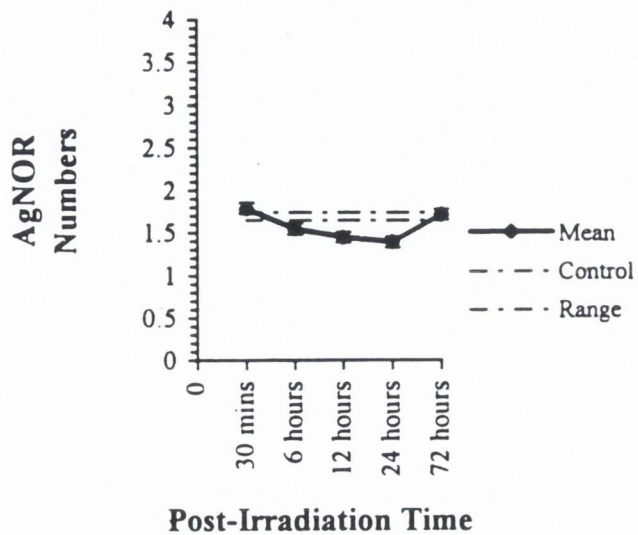
AgNOR Area vs Post-Irradiation Time *Muscle Externa*

5Gy



AgNOR Numbers vs Post-Irradiation Time *Muscle Externa*

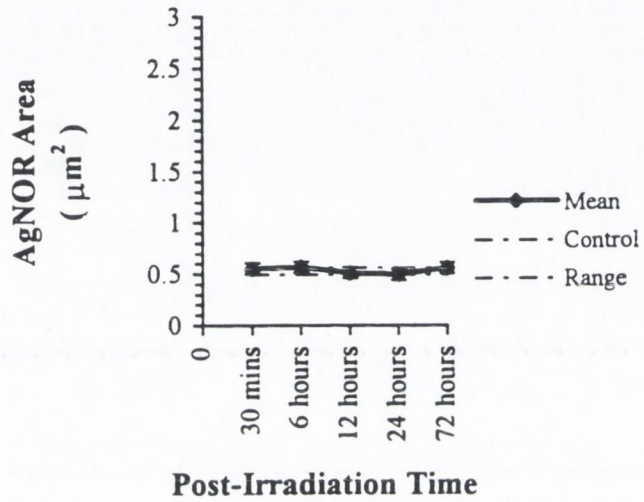
5Gy



Graph 46

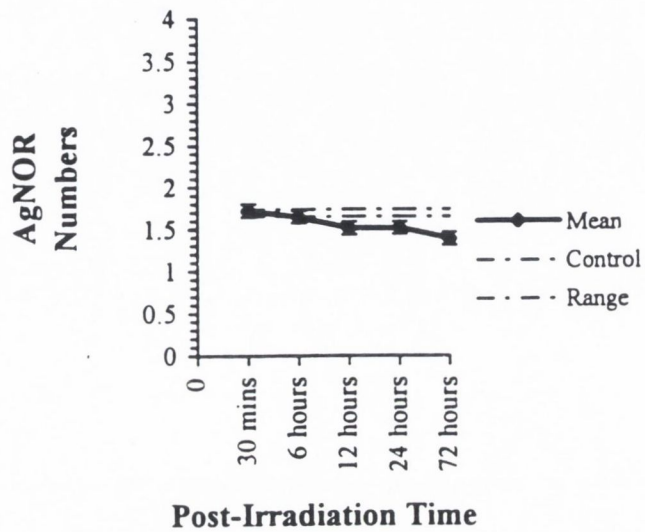
AgNOR Area vs Post-Irradiation Time *Muscle Externa*

10Gy



AgNOR Numbers vs Post-Irradiation Time *Muscle Externa*

10Gy

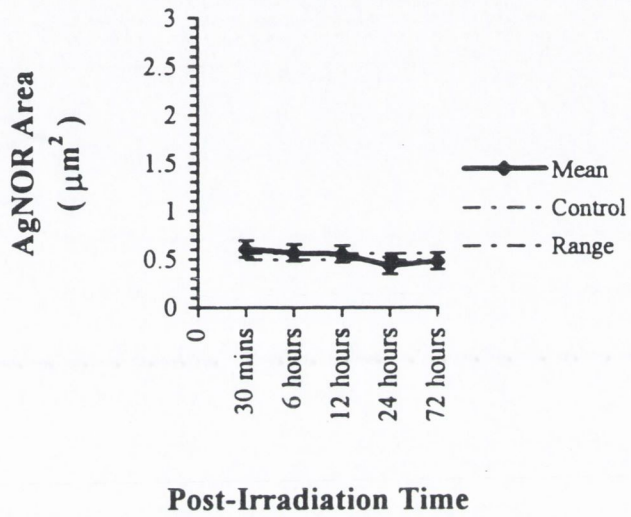


Graph 47

AgNOR Area vs Post-Irradiation Time

Muscle Externa

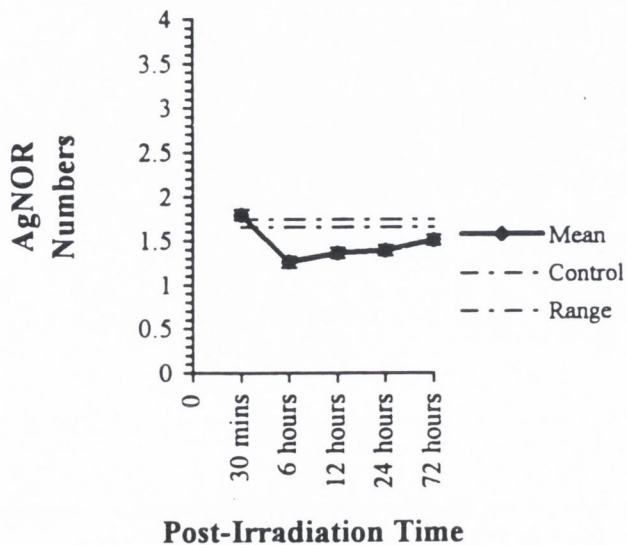
15Gy



AgNOR Numbers vs Post-Irradiation Time

Muscle Externa

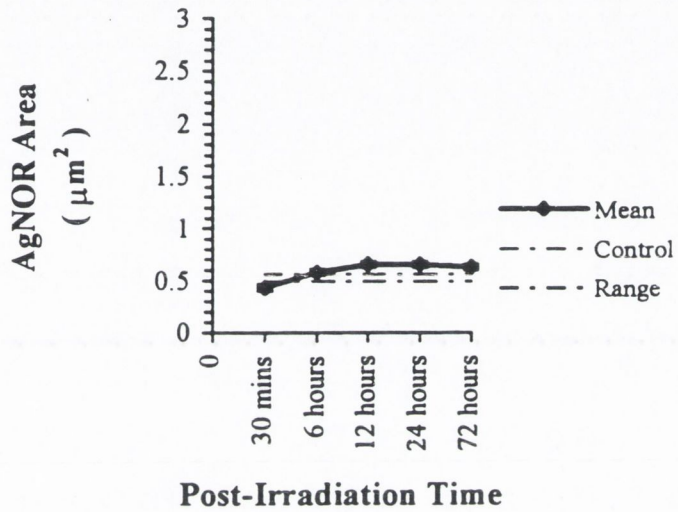
15Gy



Graph 48

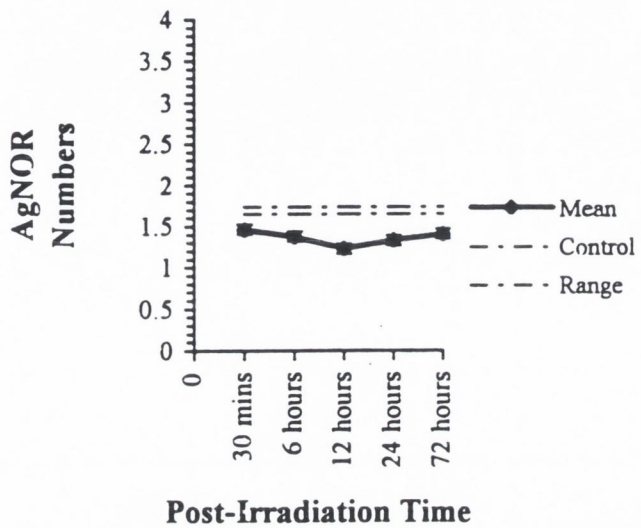
AgNOR Area vs Post-Irradiation Time *Muscle Externa*

20Gy



AgNOR Numbers vs Post-Irradiation Time *Muscle Externa*

20Gy



Part 4.

DISCUSSION

1. ASSESSMENT of CELL PROLIFERATION

The silver-staining of nucleolar organiser region associated proteins (the AgNOR technique) is one of many methods available for the assessment of cell proliferation. Although the AgNOR technique has been used for the assessment of cell proliferation in tumour tissues for almost two decades, it remains an area of controversy. Other methods of assessment of cell proliferation include mitotic counts (Akerman et al 1987), thymidine labelling (Meyer et al 1983), the S-phase labelling index (Quirke et al 1990), bromodeoxyuridine (Gratzner et al 1982) and immuno-histochemical techniques (Gerdes et al 1983).

Mitotic Counts

This was the first method to be used to evaluate cell proliferative activity in tumours. It had the advantage of being enumerated on tissue sections prepared for routine paraffin histopathology slides. However, the mitotic compartment makes up the smallest portion of cycling cells. Therefore the counting of mitotic figures has as inherent inaccuracy because of sample size. This inaccuracy is further compounded by the fact that mitotic figures cannot be easily distinguished from pyknotic nuclei. The technique was performed by tabulating the number of mitotic figures in 10 high power fields (HPFs).

Because there is variability of microscope fields between different microscopes it was difficult to show standardisation between various histopathology departments. An improvement resulted from counting mitotic figures per square millimetre of tumour profile. Unfortunately, this approach was sensitive to differences in cell size, ie: large cell tumours would contain fewer cells than a small cell tumour. Another problem encountered was section thickness. A thicker section would contain more mitotic figures than a thin section. For these reasons an even more standardised method of assessing growth fraction was introduced. This

method uses the “mitotic index” (the number of mitotic figures per 1000 tumour cell nuclei). The advantage of using the mitotic index was that cell size and section thickness parameters did not effect the result. The disadvantage was in the time taken to count the mitotic figures per 1000 nuclei.

Thymidine Labelling

This technique represents the number of radiolabelled nuclei per total number of cancer cells (Meyer et al 1983). Use of the thymidine labelling index (TLI) as an indicator of proliferative activity has several limitations.

- a. TLI shows the number of cells in the S-phase but does not give the duration of the S-phase. It is therefore possible to have a slow rate of cell proliferation but a high TLI.
- b. Some tumours display irregular growth patterns and it can be argued that the small sample taken from the tumour may not be representative of the whole lesion.
- c. Human inter-observer variations, reproducible tests and sample size may produce misleading results.
- d. The requirement of fresh tissue also limits the technique.

Bromodeoxyuridine and Flow Cytometry

The analog of thymidine is incorporated into the DNA of synthesised cells (Gratzner et al 1982). The prognostic relevance of the proliferative activity is evaluated by DNA Flow Cytometry. An advantage of flow cytometry is that DNA content can be measured in nuclei recovered from fixed and processed histological material. The most important disadvantages of flow cytometry are:

- a. The cost of the equipment required.
- b. The need to disrupt tissues so that the spatial relation of cellular subpopulations are lost.
- c. The possible inclusion of admixed neoplastic cells in samples.

Detection of Antigens Related to Cell Proliferation Using Immuno-Histochemical Techniques

Many antigens are demonstrated in proliferating cells. Two of these are used for identification of cycling cells. They are Ki-67 and proliferating cell nuclear antigen (PCNA). The use of these antibodies has become popular in many laboratories for assessment of cell proliferation in tumours (Grogan et al 1988).

When Ki-67 monoclonal antibody was first introduced, it had the disadvantage of only being used on frozen sections. However, developments of monoclonal mouse anti-human Ki-67 clone MIB1 have demonstrated that the antibody can be used on formalin-fixed paraffin embedded sections, frozen tissue sections and cell smears. The MIB1 antibody which was developed in the early nineteen nineties (Gerdes et al 1992) is the most cited Ki-67 marker in the literature. The Ki-67 nuclear protein is expressed in all proliferating cells. The expression of the protein is associated with cell proliferation. It is present during all active phases of the cell cycle (G1, S, G2 and M phases). It is absent in resting cells (G0 phase). The benefits of Ki-67 MIB1 include convenience, confidence in results and it can be used on automatic immuno-staining machines.

PCNA was tested in renal cell carcinomas (Morell-Quadreny et al 1998). Survival was significantly poorer in patients whose PCNA index was greater than 5%. The authors concluded that PCNA was a prognostic marker for renal cell carcinoma. A study was carried out on the value of PCNA as a prognostic indicator in squamous cell carcinoma of the oesophagus (Kinugasa et al 1996). The survival rate was worse in patients with high PCNA indices ($>$ or $=$ 40%) than those with low indices ($<$ 40%). However, multivariate analysis revealed that the PCNA index was not an independent prognostic factor. An assessment of the value of PCNA and AgNOR staining in endoscopic biopsies of the gastric mucosa (Irazusta et al 1998) revealed that PCNA and AgNORs seem to represent different

physiological phenomena in the cell cycle.

The use of the PCNA antibody with regard to prognosis remains controversial. In 1996 a clinical trial was carried out in Poland to test PCNA and Ki-67 values on 120 specimens from previously untreated laryngeal carcinomas (Golusinski et al 1996). PCNA and Ki-67 scores proved to be independent prognostic indicators.

Immunohistochemical methods are much more standardised than the AgNOR technique and are favoured by the majority of pathologists as the "Gold Standard" in the evaluation of cell proliferation in tumours.

The AgNOR Technique

The nature of the relationship between AgNOR quantity and cell proliferation has been reviewed in a series of projects carried out on cultured cell lines (Derenzini et al 1989, 1991). These studies reported a linear relationship between cell doubling time and AgNOR quantity. They also reported that AgNOR numbers decreased when cell proliferation was slowed down by serum deprivation. The AgNOR numbers increased to control levels when serum was added. Relating this theory to radiation it may be that there was a brief period at the early time point when the cell doubling time was increased. This was followed by a period when the cell doubling time was decreased which would lead to decreased AgNOR numbers.

The AgNOR technique has several advantages and disadvantages over existing methods of assessment of cell proliferation. The main advantages are the rapidity with which the stain can be completed, low cost and application for routine use. The main disadvantage of the technique is the lack of standardisation. Choice of tissue fixative has proved to be an important factor in AgNOR demonstration. The current optimum histological fixative for AgNOR demonstration is 10% formal saline.

The staining technique, the method of assessing the AgNORs, i.e. counting numbers, area or ratio of AgNOR numbers/nucleus, are all

variables at present in the assessment of nucleolar organiser regions. Inter-observer results present another important variable. In 1996 Srivastava et al investigated observer variation in AgNOR counts in neoplastic breast lesions. Forty five breast lesions (benign and malignant) were studied. Paraffin sections of $3\mu\text{m}$ were used and the standard AgNOR technique was used. One of the observers marked an area 1cm^2 in diameter on each slide, that contained an adequate number of well-stained cells. The slides were then coded randomly and under oil immersion at a total magnification of 1000x, AgNOR counts were then performed. The observers started counting in the top left of the field. They moved to the top right, moved down and then started counting from right to left. This pattern was carried out until 100 cells were counted. Inter-observer and intra-observer counts were assessed statistically (Bland-Altman plot). The authors stated that observer agreement in the counting of AgNORs appeared to be within acceptable limits and was statistically significant. However observer variability in AgNOR counts was high ranging from 2% -7%. In this study the authors suggested that this variability in AgNOR counts may be ascribed to the quality of the observers' eyesight, the resolution of the microscopes used, the patience and concentration of the observers in this tedious task (40 minutes/slide, manually slow) and the quality of staining with lack of artefact.

2. NUCLEOLAR ORGANISER REGIONS and their ASSOCIATED PROTEINS

Nucleolar organiser regions (NORs) are loops of ribosomal DNA (rDNA) which contain the genes which code for ribosomal RNA (rRNA) (Miller et al 1978). They are ultimately important in protein synthesis. The NOR associated proteins are a set of acidic proteins which are associated with the nucleolar organiser regions and can be selectively stained by a

silver solution (Howell and Black 1980). In 1979 Lischwe et al suggested that C23 (nucleolin) and B23 (numatrin) were the major nucleolar organiser region associated silver-staining proteins. The other known NOR associated proteins are RNA polymerase 1, 100K protein and 80K protein. Although C23 (nucleolin) is thought to be important to rRNA transcription the exact function or relationship between nucleolar organiser region associated proteins remains unknown. In order to understand the linkage between NOR associated proteins and cell activity Derenzini et al (1995) evaluated the variability of each protein independently. He concluded that in proliferating cells the high demand for ribosomal RNA (rRNA) could be satisfied by the increased phosphorylation of C23 and B23. He also stated that in cancer cell lines different quantities of C23 (nucleolin) and B23 (numatrin) were evident.

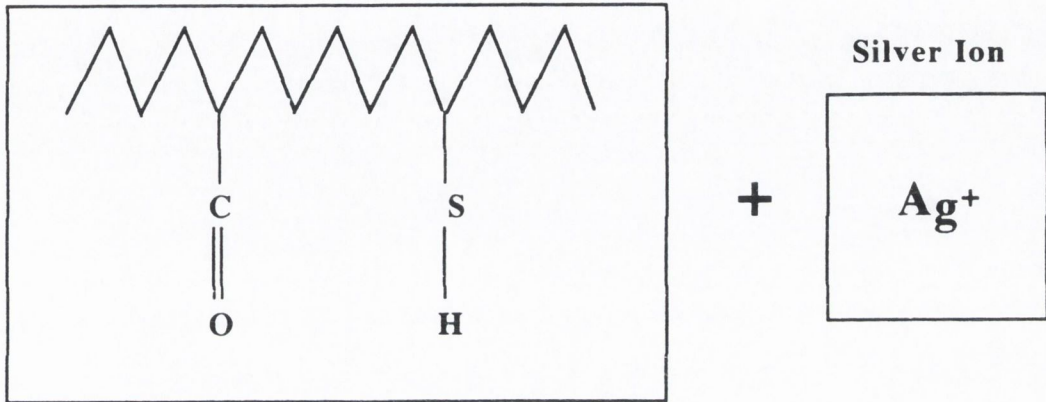
NOR Associated Protein Structure and it's Affinity for Silver Ions

When one is considering the AgNOR reaction it is important to remember that it is the NOR associated proteins that combine with the silver ions and not the actual nucleolar organiser region. The silver ions are captured by carboxyl and sulphhydryl groups on the NOR associated protein (figure 19, page 143).

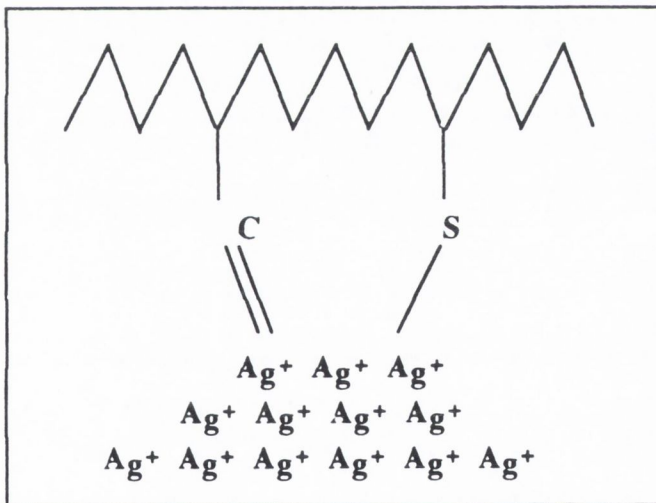
Figure 19:

BINDING of SILVER IONS to NOR PROTEINS

Nucleolar Organiser Region Associated Protein



AgNOR



Silver ions (Ag^+) are captured by Carboxyl ($C=O$) and Sulphydryl ($-S-H$) groups on the nucleolar organiser region associated proteins. Hydrogen and Oxygen are displaced.

3. MORPHOMETRY of AgNORs

Measurement has several advantages over conventional visual assessment, i.e. objectivity, reproducibility and the ability to detect changes not immediately apparent to the naked eye. Computerised image processing and analysis systems, using specialised software and hardware are capable of image capture storage and analysis. The ultimate goal of using such systems is to automate the measurement process.

Automatic image analysis of mean AgNOR area/nucleus on histological preparations is achieved using the segmentation and measurement of a digitised image (Trere et al 1989; Rüschoff et al 1990). An advantage of automatic image analysis is that it allows the parallel measurement of AgNOR number and size and a quotient of these has been used by some as measure of AgNOR content (Rüschoff et al 1990). Some workers may argue that a disadvantage of automatic image analysis is that it restricts measurements to a single focal plane. This reduces the number of countable AgNORs and precludes the absolute counts considered necessary by some workers (Crocker et al 1989).

The measurement of mean AgNOR area/nucleus using an image analysis system is a more standardised approach than manual counting in the study of AgNORs.

Comparison of Mean AgNOR Area/Nucleus with mean AgNOR Number/Nucleus

Crocker and Egan (1988), using interactive image analysis in non-Hodgkin's lymphoma, found an inverse relationship between AgNOR size and number.

Two years later Egan et al (1990) investigated the relationship between intraepithelial neoplasia of the cervix and the size and number of nucleolar organiser regions. Egan used photomicrographs to measure the

area of the AgNORs. In CIN 1 the mean AgNOR number was 2.3 and the mean AgNOR diameter was $1.28\mu\text{m}$. In CIN 2 the mean number was 3.5 and the AgNOR diameter $0.98\mu\text{m}$. In CIN 3 the mean AgNOR number was 4.7 and the mean AgNOR diameter was $0.62\mu\text{m}$. From this study it was established that there was an inverse relationship between AgNOR number and size i.e. as AgNOR the mean number/nucleus increased their mean AgNOR area/nucleus decreased.

Ofner et al in 1994 recommended that a standardised AgNOR staining method was used in all laboratories in order that results could be compared. He also reviewed the sequential quantification of AgNOR area and number during silver staining by means of an image analysis system. In this study the staining kinetics of the NOR silver staining of a human transitional cell carcinoma cell line (HOK -1) were evaluated with a image analyser at one minute intervals over a total staining period of 30 minutes. Results showed a constant increase reaching a plateau after ten minutes followed by a further area increase. By contrast, in all experiments there was a pronounced variability in the AgNOR number. The study advocated the use of AgNOR area measurement rather than AgNOR counts because of the apparent relationship between AgNOR area and staining time.

Solymosi et al (1996) investigated the value of the AgNOR technique using an image analyser in thyroid cytopathology. The AgNOR technique was applied to a total of 51 benign and malignant thyroid smears. The number and area of the AgNORs were established using an image analyser. The mean AgNOR number/nucleus and the mean AgNOR area/nucleus were both significantly higher in the malignant group. However, using the AgNOR number/nucleus value there was overlapping between the malignant and benign categories. It is interesting to note that using the AgNOR area/nucleus value there was only one case of an overlap. Because of this, the authors suggested using AgNOR area as a better discriminator between benign and malignant lesions.

4. HISTOPATHOLOGICAL APPLICATIONS of AgNORs in TUMOUR TISSUE

It has been demonstrated in neoplastic lesions that NORs (nucleolar organiser regions) are sites of ribosomal DNA (rDNA) transcription (Rüschoff et al 1990). Parallel investigations between the AgNOR technique and other indices of cell proliferation have shown that the quantity of silver-stained NOR associated proteins (AgNORs) is proportional to the proliferation of neoplastic cells. The other indices of cell proliferation reviewed in parallel with AgNOR assessment were Ki-67 scores (Hall et al 1988; Rüschoff et al 1990), bromodeoxyuridine labelling index (Tanaka et al 1989) and proliferating cell nuclear antigen (PCNA) scores (Pich et al 1995). Therefore it may be reasonable to state that AgNORs are directly related to ribosomal DNA (rDNA) transcription and cell proliferation in tumour tissue sampled.

The primary emphasis for the use of AgNOR staining has been in tumour tissues. Numerous tumours including non-Hodgkin's lymphomas, malignant melanomas, breast and colonic carcinomas have been studied using the AgNOR technique. Unfortunately in some tumours i.e.. colonic carcinomas, it has been found that although there is statistical differences between benign and malignant lesions a large overlap in counts exists. This precludes this technique as an accurate method of distinguishing between benign and malignant lesions. The counting of AgNORs in tumour appears to be of prognostic value rather than differentiating between benign and malignant lesions. Joyce et al (1992) compared AgNOR counts with the established Duke's staging of grade of tumour and concluded that AgNOR counts are highly predictive of patient survival. The controversy continued when studies such as that of Adachi et al (1995) suggested that AgNOR counts in colorectal tumours have no prognostic value.

In 1992 Paridaens et al reviewed the use of the AgNOR staining technique for prognostic value in conjunctival malignant melanomas. On this study the authors produced data on 46 patients with invasive malignant melanoma of the conjunctiva. The 46 cases were divided into two groups. The first group (A) was composed of 14 cases of malignant melanomas. All of these patients died because of the disease within 5 years. In the second group (B) 32 patients survived beyond 5 years. The mean AgNOR count in Group A was 7.03 and the count in Group B was 7.15. The authors concluded that because there was no significant difference in AgNOR counts between the two groups the AgNOR technique had no value in predicting prognosis for patients with conjunctival malignant melanoma.

In 1993 Seven et al reported on AgNOR counts in the rat stomach and their relationship to metaplasia, dysplasia and carcinoma. The study investigated AgNOR numbers in adenocarcinoma, dysplasia and intestinal metaplasia. The technique demonstrated differences in AgNOR count between normal mucosa and other lesions including adenocarcinoma, dysplasia and intestinal metaplasia. However the author reported an overlap in AgNOR counts between adenocarcinoma, dysplasia and metaplasia.

5. HISTOPATHOLOGICAL APPLICATIONS of AgNORs in IRRADIATED TISSUE

A study to investigate variations in AgNORs following irradiation was performed by Schwint et al in 1993, who reported a decrease in AgNOR numbers/nucleus and an increase in mean AgNOR area/nucleus following irradiation of squamous cells from the foot of a rat. This may be because the transcription of rDNA to rRNA was inhibited by radiation resulting in less active NORs and therefore less NOR associated proteins (AgNORs). Because there were fewer proteins available post-radiation their molecular structure may have required them to clump together to

form a stable entity.

A review of the effect of radiation on mean AgNOR numbers/nucleus in small intestine (jejunum) was carried out in 1997 (McDermott MPhil 1997). In general AgNOR numbers decreased post irradiation. This decrease, thought to be the result of inhibition of transcription, was more prevalent in the cryptal epithelium compartment.

6. RESEARCH and ANALYTICAL RESULTS

Before proceeding with irradiated specimens an evaluation of the mean AgNOR area/nucleus within the various compartments of mouse jejunum in control tissue was completed. The mean AgNOR area/nucleus ranged from $0.46\mu\text{m}^2$ in the upper villus to $1.2\mu\text{m}^2$ in the cryptal epithelium. It may be suggested that the high level of mean AgNOR area/nucleus in cryptal epithelium ($1.2\mu\text{m}^2$) reflects both a high level of cell proliferation and transcriptional activity. Proportionately the mean AgNOR area/nucleus in the upper villus ($0.46\mu\text{m}^2$) may also reflect a low level of cellular proliferation and transcriptional activity. It may be possible that proliferating cells such as cryptal epithelium have a different NORAP structure to non-proliferating cells, i.e. more carboxyl and sulphhydryl groups available for silver ion capture. This would explain the larger AgNOR area/nucleus values in proliferating cells. There was no significant difference in mean AgNOR area/nucleus between pre-stressed non-irradiated controls (shams) and non-stressed, non-irradiated controls in any of the compartments studied.

The second part of the current study was to assess the effect of gamma radiation on the mean AgNOR area/nucleus within the various compartments of mouse small intestine (jejunum) at timed intervals, post-irradiation, using an automatic image analysis system. These intervals ranged from 30 minutes to 72 hours. The doses of gamma radiation were

5Gy, 10Gy, 15Gy and 20Gy. Results from this part of the study showed that there was a general increase in the mean AgNOR area/nucleus in the cryptal epithelium compartment, post-irradiation. This increase was both dose and post-irradiation time dependent. There was no significant change in mean AgNOR area/nucleus in any of the other compartments under review. Before considering the significance of this increase in mean AgNOR area/nucleus it may be worth while to debate the effect of gamma radiation on DNA which is an important part of the nucleolar organiser region.

It may be that when the rapidly proliferating cryptal epithelial cells were exposed to gamma radiation DNA was damaged to varying degrees depending on dose. This damage may have been repairable or non repairable depending on the type of strand break. When a single strand break occurred the site of the damage may have been identified and the break easily repaired by annealing of the broken ends. If base damage occurred on a single strand, enzymic excision would have taken place and the in-tact complementary strand of the molecule would have provided a template upon which to reconstruct the bases in the correct sequence. If a double strand break occurred damage to the DNA would have been much more significant rarely resulting in repair. This damage to the DNA of the cell may have resulted in inhibition of cell proliferation and therefore less AgNORs.

When a 5Gy dose was administered the type of DNA damage may have been repairable (single strand break). This may be why the mean AgNOR area/nucleus values almost returned to control values at 72 hours. When the larger doses of radiation were administered (10Gy, 15Gy and 20Gy) double strand breaks to the DNA may have occurred and transcription of rDNA to rRNA may have ceased. This may have been why the mean AgNOR area/nucleus increased initially and did not return to control values.

In this study the AgNOR area/nucleus within the cryptal epithelium increased post-irradiation. There are two possible suggestions as to why this has occurred:

1. Because there was a decrease in proliferation post-irradiation there were less AgNORs. The remaining AgNORs (silver-stained NOR associated proteins) may have unfolded structurally due to the radiation and possibly exposed more carboxyl and sulphhydryl groups which have an affinity for silver ions. This would then have resulted in an increase in AgNOR surface area.
2. Existing NOR associated proteins may have unfolded due to radiation and then bound together (clumped), to form a more stable entity. This could also be a reason for the increased surface area of the AgNOR.

Stable and permanent cell populations such as connective tissue and muscle cells as well as ageing cells such as the upper villus epithelial cells, did not show any significant change from control values. This may be because little or no transcription or cellular proliferation occurred in these compartments.

In the third part of this study the mean AgNOR area/nucleus data was compared with results from a previous study, i.e. mean AgNOR number/nucleus post-irradiation in the mouse small intestine (McDermott MPhil 1997). This comparison, illustrated in graph form, of mean AgNOR area/nucleus and mean AgNOR number/nucleus post-irradiation, demonstrated that in general there was a significant inverse relationship within the cryptal epithelial compartment, i.e. as the mean AgNOR area increased the mean AgNOR number/ decreased. This inverse relationship was only evident within the cryptal epithelium compartment. The upper villus, lower villus, villus lamina propria, lamina propria and muscle externa did not exhibit this relationship. The decrease in the mean AgNOR number/nucleus was thought to be because of radiation induced cell proliferation inhibition. The increase in the mean AgNOR area/nucleus

may be due to possible changes in nucleolar organiser protein structure as previously described. These findings support the study of Schwint (1993) who also reported on an inverse relationship between AgNOR area and AgNOR number post-irradiation.

In the final section of the study, control, post-irradiated and tumour samples were examined using the standard emission mode and back-scattered mode of a scanning electron microscope. Control and post-irradiated small intestinal samples were regular in structure and they appeared as uniform smooth edged regular structures. In the malignant tumour (adenocarcinoma) rapidly proliferating cells exhibited AgNORs with a bizarre three-dimensional structure resembling icebergs. This maybe because the NOR associated proteins are being produced at an abnormally rapidly rate. Because of this the NOR associated proteins may be incorrectly formed structurally, present in the incorrect amount or possibly in an incorrect ratio to each other. This may have lead to abnormal quantities of free carboxyl or sulphhydryl groups and would explain the abnormal silver deposits (bizarre structures) observed in this study. These results were similar to the study of Thiebaut in 1984 who looked at cultured tumour cells using the back-scattered mode of the scanning electron microscope. This study also described bizarre structures. Similar results were also shown by Imamura in 1993 who produced a three dimensional display of tumour cells using the confocal laser scanning microscope.

When one examines the photographs of the current study it becomes clear why there has been so much debate into the usefulness of the AgNOR technique. In the 1980's when researchers were counting AgNORs they may actually only have been enumerating the tips of the bizarre structures (icebergs). This may explain inconsistencies between studies and observers. Later in the 1980's and early 1990's researchers examined AgNOR area. From the current scanning electron microscope photographs it can be seen

that they were actually measuring only a single cut section through the "peaks" of the tumour AgNORs. Obviously the AgNOR area measured by the image analyser would depend on the level of the cut as this instrument only measures the area of the AgNOR on one focal plane. Presumably this would explain the variation in observations and conclusions drawn about the usefulness of the technique.

7. POTENTIAL CONSIDERATIONS for FUTURE RESEARCH

Existing methods of assessing radiation damage to tissues include crypt counting (Withers et al 1969) and the use of a Morphological Index (MoI) to assess total structural damage (Carr et al 1992). The assessment of radiation damage using the Morphological Index has also been applied to tissues irradiated by different forms of radiation (Carr et al 1996). Neutron and X-ray radiations were compared. It was shown that the response of individual cell types could not be predicted from knowledge of the change in crypt numbers. Neutron treatment led to more damage to the neuro-muscular components of the wall whereas X-irradiation produced vascular changes. Having established that, in the cryptal epithelium, there was an inverse relationship between mean AgNOR area/nucleus and mean AgNOR number/nucleus, it may be possible in future to use this information as an additional potential factor in the Morphological Index equation (MoI).

It would be very interesting to measure the surface area of the three-dimensional image of silver-stained NOR associated proteins in various malignant lesions using the back-scattered mode of the scanning electron microscope. It would also be interesting to observe the structure of pre-irradiated and post-irradiated tumour samples using the combination of the AgNOR technique and the back-scattered mode of the scanning electron microscope. If the surface area of the tumour AgNOR is related to the activity of the tumour then this technique may give additional information on prognosis.

In a study by Meehan et al (1993) a range of cytological samples were prepared from fresh surgical specimens of breast. This was accomplished by scraping fresh breast samples with a scalpel and smearing

them on a clean slide. AgNOR stains were carried out and they were then scored by a subjective scoring technique. Although the accuracy reached 90% there was still overlap between benign and malignant parameters. The authors felt from a diagnostic point of view AgNORs alone were probably not useful in differentiating between benign and malignant breast lesions.

It would be of great interest to examine similarly prepared breast specimens, as that of Meehan, under the scanning electron microscope, using the same method as described in the current research. If abnormal peaks and bizarre structures were found to be present in only malignant lesions then, when looking at the overlap specimens, if similar abnormalities were present this could presage malignant transformation.

Part 5.

CONCLUSION

From this study it can be concluded that:

1. There was variation in the mean AgNOR area/nucleus within the various compartments of the control small intestinal samples (jejunum), i.e. the AgNOR area/nucleus ranged from $0.46\mu\text{m}^2$ in the upper villus to $1.2\mu\text{m}^2$ in the crypt epithelium.
2. There was a significant increase in mean AgNOR area/nucleus in the cryptal epithelium compartment following various doses of gamma radiation. This increase was not present in any of the other compartments studied.
3. There was an inverse relationship between the mean AgNOR area/nucleus and the mean AgNOR number/nucleus within the cryptal epithelium (post-irradiation). This phenomenon was not present in any of the other compartments reviewed within the small intestine.
4. The study of the structure of silver-stained nucleolar organiser regions in neoplastic tissue, using the scanning electron microscope, has shown images of tumour AgNORs as bizarre irregular structures. This phenomenon invites further research that could lead to important additional information becoming available for patient prognosis.

Part 6.

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