THE EARLY AND LATE EFFECTS OF LOW LEVEL LASER IRRADIATION ON HUMAN OSTEOSARCOMA CELLS

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A thesis submitted in partial fulfilment

of the requirements for the degree of

MASTER OF DENTAL SCIENCE

(Orthodontics)

Discipline of Orthodontics

School of Dental Studies

Faculty of Dentistry

University of Sydney

Twentieth of September, 1999

DEDICATION

This thesis is dedicated to my wife, Mimi, for her endless support and love. It would not be possible to finish the course without her tolerance, encouragement, understanding and assistance. She worked as hard as I did for the master degree. She has stood by me for the last three years as she did during my studies in Melbourne. Her love is strongly felt and appreciated. I also dedicate my work to my parents and siblings in Hong Kong for their trust and continuous support. I was sorry that I could not be with my parents when they were sick. Moreover, I could not share their feeling of uncertainty during the Hong Kong change-over in 1997. This thesis is also dedicated to the memory of my father-in-law, S. H. Lau (1933-1998).

ABSTRACT

Low Level Laser Therapy (LLLT) has been used in treating many clinical conditions. It has been reported to have multiple clinical effects including promotion of wound healing and bone healing. It has also been reported to stimulate cellular functions. However, the effects of wavelength, beam type, energy output, energy level, energy intensity and exposure regime of LLLT remain unexplained. There are no reported studies of LLLT on human osteoblastic cell lines. The aim of the study was to investigate the early and late effects of LLLT on protein expression and alkaline phosphatase (ALP) activities of the human osteosarcoma cell line, SAOS-2. Cells were seeded into 96 well plates before laser irradiation. The cells were irradiated daily for 4 -7 days with a GaAlAs continuous wave diode laser (830nm) with a net output of 90mW. Duration of irradiation was 3, 5.5, 11, 22 and 44 seconds with energy level 0.27, 0.50, 0.99, 1.98 and 3.96J. The corresponding energy intensities were 0.017, 0.032, 0.062, 0.124 and 0.249J/mm^2. Cells extracts were obtained one hour (for early effects) or 24 hours (for late effects) after two and four irradiations. SDS-PAGE was performed on the cell extracts. ALP activity analysis was performed one hour (for early effects) or 24 hours (for late effects) after each laser irradiation. No significant early or late effects of laser irradiation on protein expression and ALP activity were found. There was, however, a trend for laser irradiation at high energy levels (0.99–3.96J) to be associated with a decrease in ALP activities one hour after irradiation. Conclusions: 1) No significant early or late effects of multiple laser irradiations with a GaAlAs continuous wave diode laser, wavelength: 839nmn, output: 90mW, power density: 0.017-0.0249J/mm^2 on protein expression of a human osteosarcoma cell line SAOS-2 were found. 2) No significant early or late effects of multiple laser irradiations with a GaAlAs continuous wave diode laser, wavelength: 839nmn, output: 90mW, power density: 0.017-0.0249J/mm^2 on ALP activity of a human osteosarcoma cell line SAOS-2 were found. 3) There was a trend for laser irradiation at high energy levels to be associated with a decrease in ALP activity one hour after irradiation. However, a general dose-related response could not be identified.

ACKNOWLEDGMENTS

Acknowledgment of those who have helped with this thesis should, I feel, begin with my dear colleagues and friends. This thesis would not have been possible without the their support and assistance. In particular, I wish to acknowledge the tremendous support, encouragement and guidance from my supervisors, Professor M. Ali Darendeliler and Dr. Neil Hunter. Their patience and advice during the last three years is deeply appreciated. I would also like to thank Dr. Geoff Wright for his guidance and friendship.

I am particular grateful to my colleagues, Michèle Fong, Yvonne Poon, and Steve Stramotas whom have shared my laughter and sorrow in the last three years. I would also like to thank Sherryn Clinton, Jill Tayler, Luke Leung and Antonio Lee for their friendship and support.

I would also like to thank the colleagues and staff in the Institute of Dental Research. I am grateful to Julia Philips, Peter Yun and Derek Harty for their technical advice and assistance. Last but not the least, I would like to thank my research partner, Angela Coombe for her understanding during the difficult time.

The contributions made by the Faculty of Dentistry, University of Sydney and Australian Society of Orthodontists Foundation for Research and Education are deeply appreciated. I would like to thank the Department of Oral Pathology, Westmead Centre for Oral Health for the provision of the osteosarcoma cell culture. I am grateful to the Liberato-Lasers, Demark for the loan of the P-LaserSystem.

Mostly, I thank my wife, Mimi, and my family in Hong Kong who were always encouraging, helpful and tolerate of my total distraction from the normal routines of home life and recreation during the master course.

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CHAPTER ONE

INTRODUCTION

The surgical, ophthalmological, and dermatological applications of high power lasers are well known (Colvard and Pick 1993). The potential preventive and therapeutic hard-tissue applications of CO_2, ND:YAG and argon lasers in dentistry are also explored (Gonzalez *et al.*, 1996). Lasers at powers of low magnitude (Low Level Laser) have also been used in the laboratories and clinics for nearly 30 years. Clinically, therapy utilising low level lasers, Low Level Laser Therapy (LLLT), is being used in many medical conditions including arthritis, tendonitis, pain management, soft tissue wounds, sport injuries, nerve repair, tinnitus, immune modulation (Basford 1995). LLLT has also been used in dentistry (Walsh 1997a, Walsh 1997b). The effects of LLLT including pain relief, promotion of soft tissue and hard tissue healing (Trelles & Mayayo 1987, Takeda 1988, Lee *et al.*, 1993, Khuller *et al.*, 1996, Saito & Shimizu 1997, Harazaki *et al.*, 1998) have possible orthodontic applications eg. pain reduction during orthodontic treatment, improvement of bone healing, increase in rate of tooth movement, reduction in root resorption. LLLT is widely used in many clinical conditions, however, its mode of action is not fully determined. The effects of different wavelengths, beam types, power outputs, energy levels, energy intensities, exposure regimes of LLLT on different types of tissue remain elusive.

1.1 Laser beam generation

LASER is an acronym for Light Amplification by Stimulated Emission of Radiation (Midda & Renton-Harper 1991). Normal light is created by photons emitted when electrons are excited and then return to their stable level (Fig. 1.1). Normally, most of the electrons of an atom will be on the lowest energy level which is called the ground level (Fig. 1.1a). If the outside energy e.g. light energy is absorbed by the atom, the electrons will become excited (Fig. 1.1b). The excited electrons are unstable and quickly return to a stable level, sending off photons (Fig. 1.1c). This process is called spontaneous emission (Kert & Ross 1989). The size of the change in energy is directly related to the wavelength of the absorbed

and emitted light. The greater the energy change, the shorter the wavelength and vice versa.

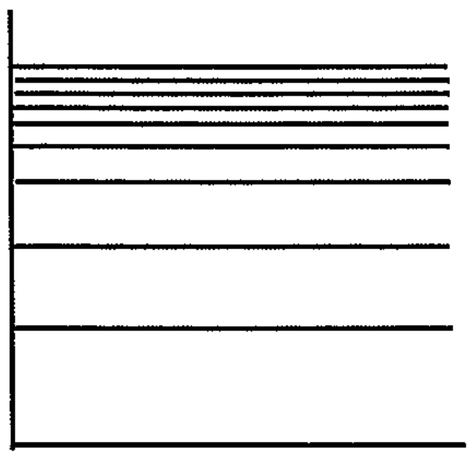

Figure 1.1a Energy at ground state level

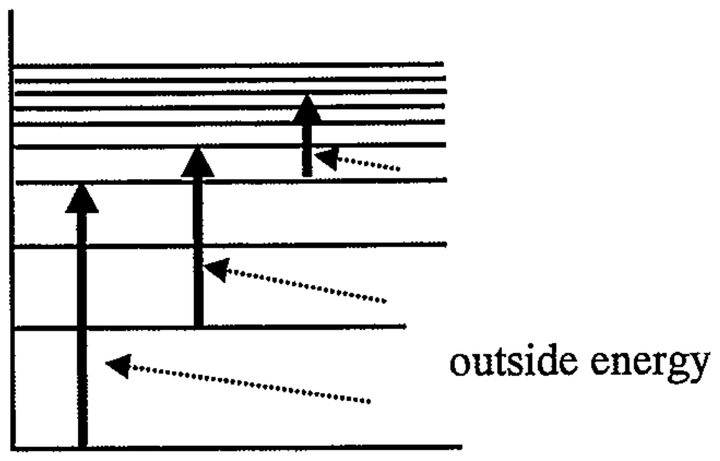

Figure 1.1b Absorption of energy

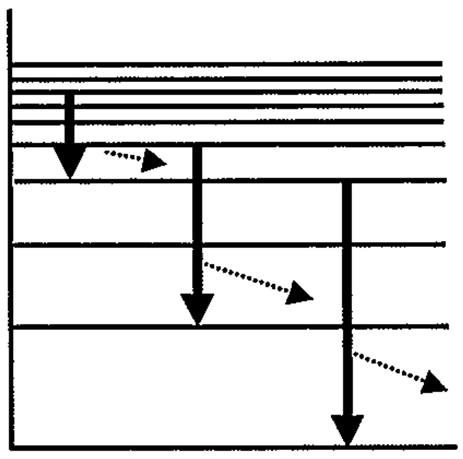

Figure 1.1c Emission of photons

Figure 1.1. Atom absorption and spontaneous emission

Laser is the result of stimulated emission occurring when an incoming photon (Fig 1.2a) stimulates an excited atom. The excited atom then returns to a stable energy level (Fig. 1.2b) and emits a photon identical to the incoming one (Fig. 1.2c). The incoming photon must have a wavelength that is identical to the energy which the atom will release when the electron falls from the excited level to a more stable one (Fig. 1.2).

Figure 1.2a
Incoming photon

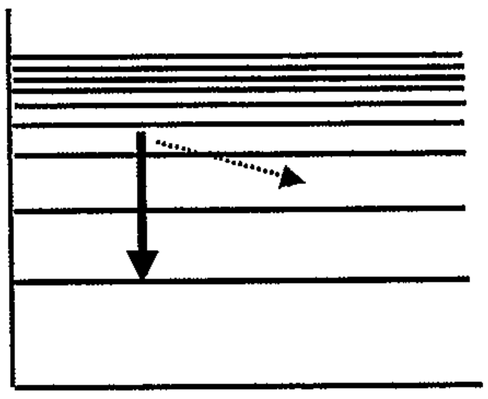

Figure 1.2b Excited electron returns to a stable level

Figure 1.2c Incoming photon and stimulated photon

Figure 1.2. Stimulated emission

1.2 Laser sources

A laser beam is generated when an external power source stimulates a chamber of a laser medium - solid, liquid, gas, or semiconductor. The choice of material determines the laser's characteristics eg. wavelength. The resulting stimulated emission of photons resonates between the mirrored ends of the laser chamber. These bouncing photons further excite other atoms in the laser medium. Momentum builds up until a highly concentrated beam of light passes through the partially transmissive mirror at one end of the chamber. The individual light wave generated is identical to an ordinary light wave (Kert & Rose 1989). Figure 1.3 shows the principle components in a laser probe. The excitation mechanism supplies the energy. This can either be in the form of intense light or electricity.

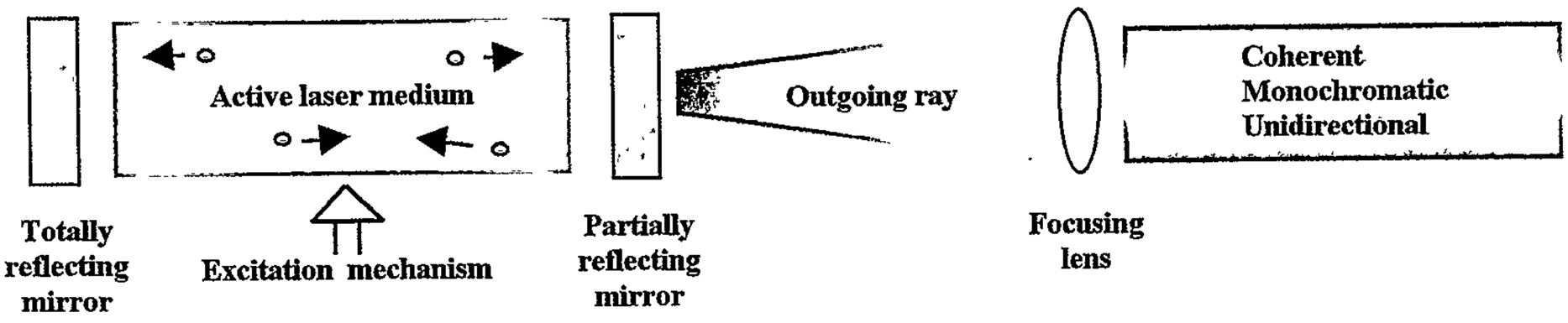

Figure 1.3. Principle components in a laser probe

1.3 Beam types

Light emitted from a laser can either be in the form of a continuous wave beam or a pulsating one (Karu 1998). A continuous beam has a laser's power output,

measured in milli-Watts (mW) which remains constant throughout a given time period (Fig. 1.4). If the continuous beam is interrupted, a pseudo-pulsating beam is created (Kert & Rose 1989). The individual pulsations have the same power output as the continuous beam from which they are derived (Fig. 1.5)

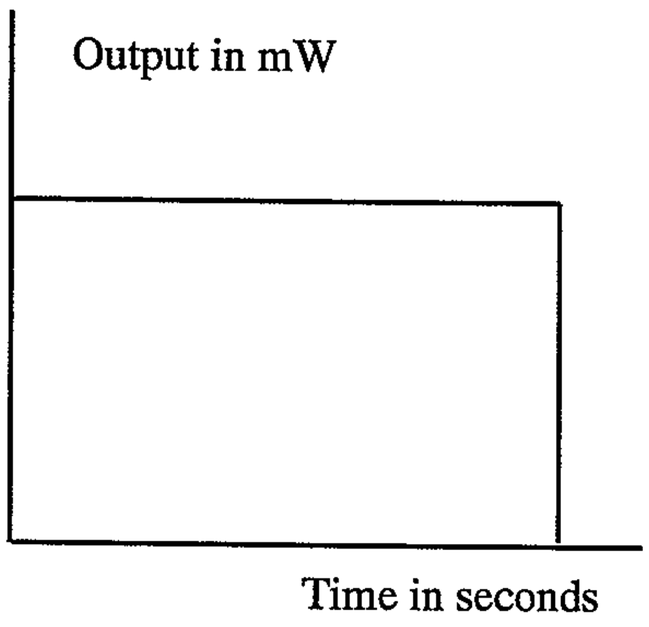

Figure 1.4. Continuous beam

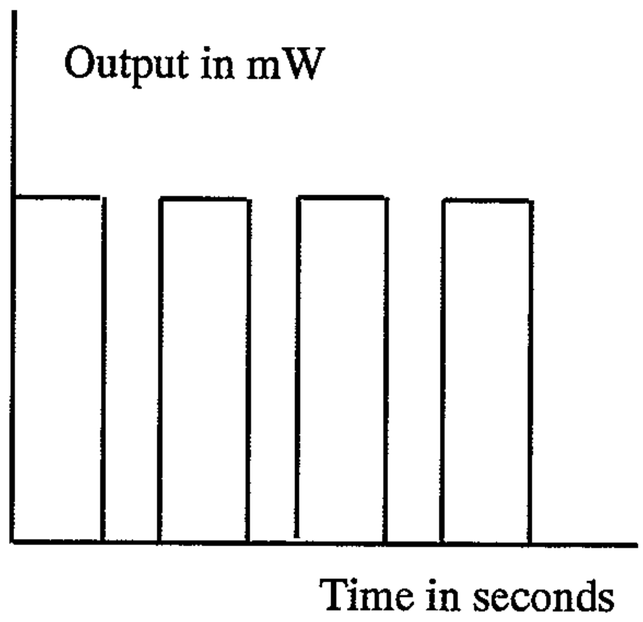

Figure 1.5. Pseudo-pulsated beam

In the true pulsed lasers, the light itself pulsates. In these lasers, charging up occurs until a particular energy level is reached (Fig. 1.6). At this point the laser is emitted.

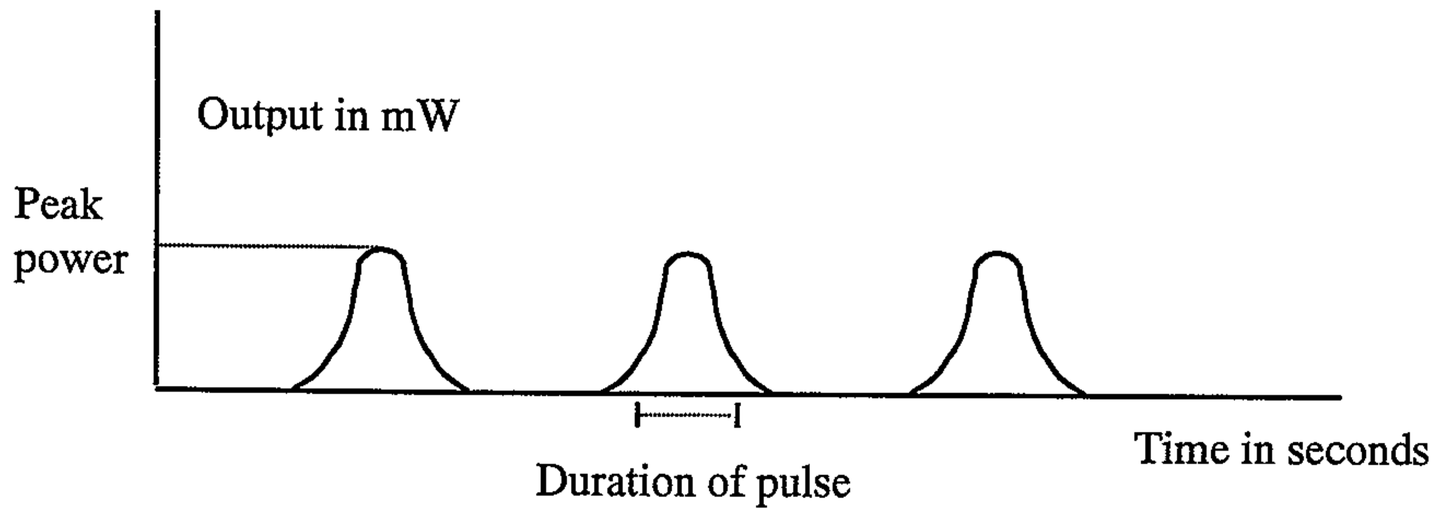

Figure 1.6. True pulsed laser

Pulsed lasers of this type usually have a high maximum power output (peak power). The duration of the individual pulsations ranges from nanoseconds to milliseconds. Pulsations can also be expressed in terms of pulses per second, i.e. pulse frequency. The mean energy output which is usually much lower than the peak power is used for calculation of energy level and intensity in a pulsed laser (Kert & Rose 1989).

1.4 Characteristics of laser

Laser has three characteristics which make it different from normal light (Karu 1998)

a) Monochromaticity

Depending on the medium, the photons emitted in a laser beam can all have the same wavelength. In other words, a laser beam consists of only one colour. The spectral band created by a laser is very narrow but dual or multiple wavelength emissions are possible.

b) Coherence

All photons emitted in a laser are spatially and temporally in phase.

c) Unidirectionality

A laser beam has small beam divergence. The output intensity is maintained even after the laser travels a long distance. Some spreading of the laser beam does take place. A typical divergence for laser light is 1 milliradian. The beam diameter increases one unit for every 1000 units or it increases by 1 mm across a distance of 1 metre. However, if the laser beam comes from a single point source, typical of diode lasers, the beam must first be collimated by a focusing lens (Kert & Rose 1989).

1.5 Energy output

Laser power is expressed as output measured in Watts. Laser energy level is expressed in Joules. Energy is a product of mean output and beam duration (Basford 1995) i.e. Energy (Joules) = Mean output (Watts) x Time (seconds).
In most circumstances, the laser dosage is also expressed as power intensity or density (mW/mm^2), or energy intensity or density (J/mm^2 or J/cm^2) (Karu 1998).
When laser energy impacts a targeted tissue, two factors determine the effect the laser energy will have on the tissue. The first factor is the absorptivity of the tissue medium. The second factor is the wavelength of the laser energy that is to be absorbed in the tissue. Kert and Rose (1989) described maximum degree of effectiveness (DE) as an expression of the proportion of the delivered laser output which was actually absorbed by the treated tissue. Therefore,

Net Energy (Joules) = Mean output (Watts) x Time (seconds) x DE

In general, the wavelength of the laser and the absorption coefficient of the tissue will significantly determine the effect that the laser energy will have on, or within the targeted tissue (Colvard & Pick 1993).

1.6 Low Level Laser

Two categories of lasers are used in medicine and dentistry (Midda & Renton-Harper 1991). "Hard" lasers, with high energy output, have been extensively used for surgical applications (Miller & Truhe 1993, Pick 1993). "Soft" lasers are athermal, low energy lasers (Midda & Renton-Harper 1991). These are at powers that are orders of magnitude smaller than hard lasers. Low intensity, low level, and low power lasers which emphasise the athermal, low energy effect of the lasers are interchangeable terms for soft lasers (Basford 1995). Recently developed low level lasers generally utilise semi-conductor laser diodes (Midda & Renton-Harper 1991). Table 1.1 lists the laser medium for low level laser.

Table 1.1. Low level laser medium in clinical use (Basford 1995)

Laser medium	Abbreviation	Wavelength(s) nm
Helium neon	HeNe	632.8
Gallium aluminium arsenide	GaAlAs	820, 830
Gallium arsenide	GaAs	904
Neodymium:yttrium-aluminium-garnet	Nd:YAG	1064
Carbon dioxide	CO_2	10600
Argon	Ar	488, 514
Krypton	Kr	521, 530, 568, 647
Ruby		694

1.7 Low Level Laser Therapy

Low Level Laser Therapy (LLLT), is the commonly used term to describe laser treatment using low level laser (Kert & Rose 1989, Walsh 1997a, Walsh 1997b). This therapy is essentially used to aid in tissue regeneration, primarily employed to relieve pain, reduce inflammation and oedema and accelerate healing (Midda & Renton-Harper 1991). In practice, LLLT typically involves the delivery of ≤ 1-4

J/cm^2 to the treatment site utilising lasers with output powers between 10 mW and 90 mW. Many reports support the effectiveness of LLLT used in multiple clinical conditions (Kert & Rose 1989, Sexton & O'Hare 1993, Gray *et al.,* 1994, Basford 1995, Bhagwanani *et al.,* 1996). However, early clinical work was usually anecdotal, unblinded, poorly controlled and incompletely reported (Basford 1995). Table 1.2 lists some of conditions reported to be treated with LLLT with some success.

Table 1.2. Conditions reported to be treated with LLLT (Kert & Ross 1989, , Sexton & O'Hare 1993, Gray *et al.*, 1994, Basford 1995, Bhagwanani *et al.*, 1996)

Bactericidal effects	Patellofemoral pain
Buerger's disease	Periarthritis (frozen shoulder)
Haemangiomas	Pruritus
Headaches (vascular and muscular)	Pulmonary tuberculosis
Immune modulation including leukaemia	Soft tissue wounds
Inflammatory arthritis	Sports injuries
Musculoskeletal pain including TMD	Sympathetic nervous system dysfunction
Nerve repair	Temporomandibular joint disorder
Neuropathic and oro-facial pain	Tendonitis
Osteoarthritis	Tinnitus

1.8 Treatment parameters

Despite similarities of dose and a convergence in laser choice, significant differences exist between treatment approaches (Tab. 1.3).

Table 1.3. Treatment parameter variations (Basford 1995)

Parameters	**Variation**
Wavelength	Typically 632.8, 820, 830 or 904 nm
Powers	Average powers 10-90 mW
Waveform	Continuous wave, pulsated (1-4,000 Hz)
Dosage	1-4 J/cm^2
Regime	Treatments from a few to 30 seconds on a daily or alternate day schedule
Spot size	from ≤ 1 mm^2 to defocused and scanned beams covering the treatment area

Kert & Rose (1989) summarised their treatment protocol and defined their dosage principles. According to Kert & Rose (1989), correct dosages of LLLT are of vital importance if optimum treatment results are to be achieved. An assumption is made that the treated tissue will be affected no matter what dosages are used. The effect is called a tissue reaction. When sufficient energy has been delivered to the targeted tissue and causes a noticeable tissue reaction in the patient, it is then called a treatment reaction. It is recommended that a dosage should be sufficient to cause a treatment reaction. Clinically it is called minimal reaction dose. It is essential to attain this dose each time a treatment is administered. Table 1.4 lists the clinical effects of LLLT with recommended dose of laser (Kert & Rose 1989).

Table 1.4. Clinical effects of LLLT with recommended dose of laser (Kert & Ross 1989)

Clinical Effect	Response time	Dosage at start of treatment
Muscle relaxation	Immediate	2 J
Acupuncture point stimulation	Immediate	0.1-0.5 J
Nerve stimulation	Immediate	2 J
Release of contracture	Immediate/days	5-7 J
Improved microcirculation	Minutes/hours	1-3 J
Anti-inflammatory effect related to acute inflammation	Minutes/hours	1-3 J
Anti-inflammatory effect related to chronic inflammation	Days/weeks	3-6 J
Pain relief	Minutes/hours	1-4 J
Revascularisation	Hours/days	2-3 J
Anti-viral effect	Hours/days	1-2 J
Nerve cell regeneration	Days	2 J
Healing advancement	Days/weeks	1-4 J

Kert and Rose (1989) recommend a general protocol of LLLT for most medical conditions with a history of less than 6-8 weeks (Fig. 1.7).

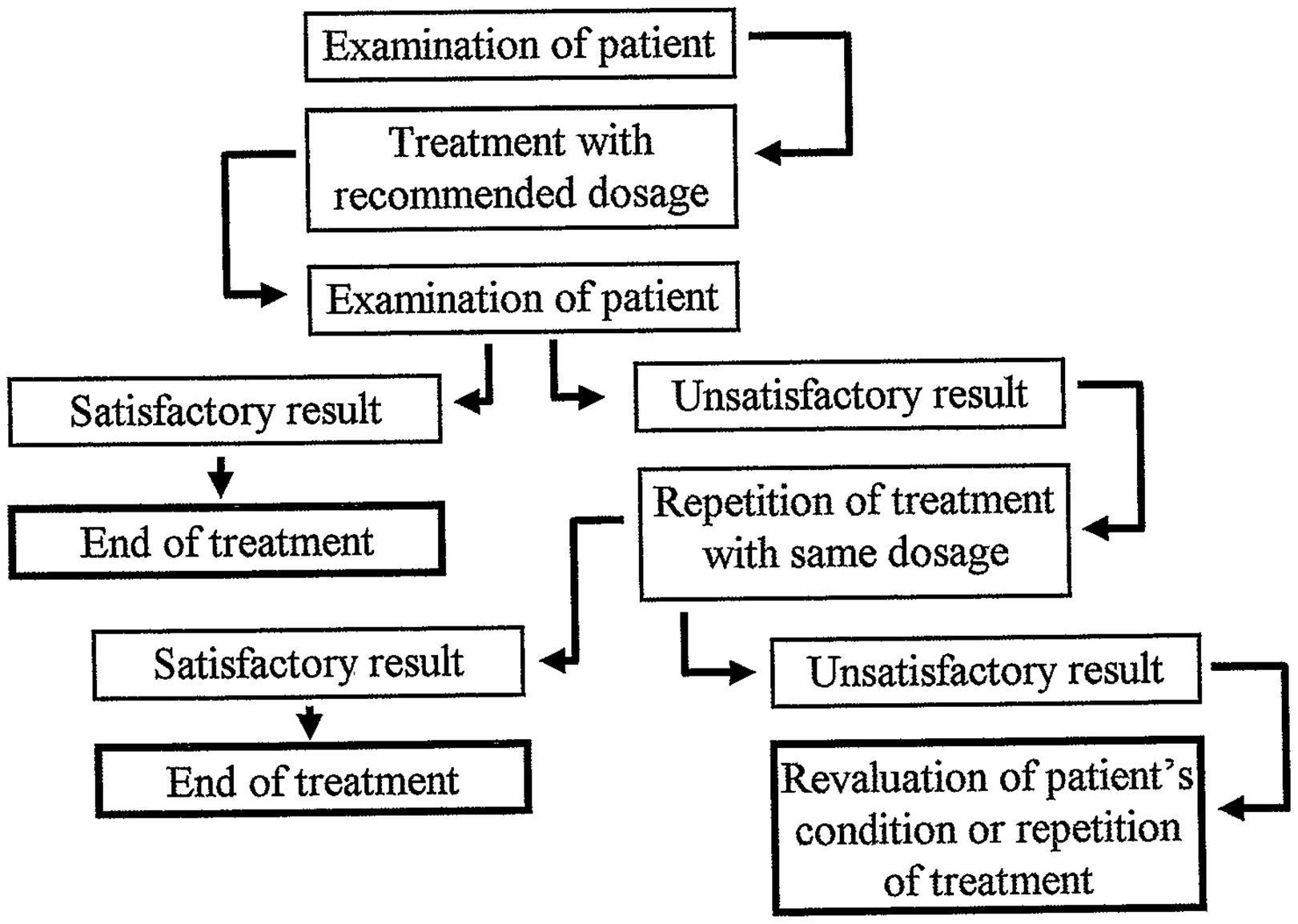

Figure 1.7. General protocol for LLLT

1.9 Current research

Numerous clinical and laboratory reports in the last decade show the effects of low level laser on cellular functions. Table 1.5 summarises the reported possible cellular functions altered by LLLT. However, many other studies show no effect of LLLT (Basford 1986, Basford 1995).

Table 1.5. Possible cellular functions altered by LLLT (Basford 1986, Basford 1995)

Cellular function	Effects	
Protein synthesis	↑	↓
Cell growth and differentiation	↑	↓
Cell motility	↑	
Membrane potential and binding affinities	↑	
Neurotransmitter release	↑	
Phagocytosis	↑	
ATP syntheses	↑	
Prostaglandin synthesis	↑	

1.9.1 Animal studies

There have been considerable numbers of animal studies published in English during the last two decades.

1.9.1.1 Soft tissue effects

Dairy cattle were used in two soft tissue wound healing studies. Ghamsari *et al.,* (1996) made perforating wounds on the surface of 32 teats in eight dairy cattle. The authors sutured the wounds with 4 different kinds of suture patterns. Half of the wounds were subjected to continuous wave He-Ne laser irradiation (Wavelength: 632.8nm, energy output: 8.5mW, energy intensity: 3.64 J/cm^2). The wounds were examined histologically and it was found that laser irradiation accelerated the healing of the perforating wounds. Ghamsari *et al.,* (1997) published another study of teat wound healing in dairy cattle. The authors made perforating wound on 16 teats in 4 cattle. The wounds were sutured with 2 different kinds of suture pattern. Half of the wounds were subjected to the same laser irradiation as in the previous study. The teat wall in the non-irradiated groups was significantly thicker than that in the irradiated group on day 7, 14 and 21. Histologically, the morphology of the epidermis in the irradiated groups resembled more closely to the normal epidermis than that of non-irradiated groups. In the irradiated groups, collagen fibres were denser, thicker, better arranged and more continuous with the existing fibres. The authors reached the same conclusion as for the previous study.

On the other hand, McCaughan *et al.,* (1985) studied four open circular wounds on the backs of each of ten guinea pigs. The wound involved excision of 1cm in diameter of full thickness skin and subcutaneous tissue and no dressings were applied. Each wound in nine guinea pigs was 1) treated with saline and peroxide, 2) argon low-power irradiation (output: 2mW/cm^2, energy intensity: 2J/cm^2), 3) washed with saline and peroxide and then irradiation and no treatment at all. The tenth guinea pig received similar treatment except with a sham laser. The ulcers were treated and measured every 2 to 3 days after the initial measurement. The authors found that argon laser exposure had no effect on the rate of wound healing in guinea pigs.

Two studies with 810nm GaAlAs diode laser on bone and cartilage changes induced in rats by joint immobilisation have been published recently (Akai *et al.*, 1997, Usuba *et al.*, 1998). Akai *et al.*, (1997) operated on the hind limbs of 42 young Wistar rats and immobilised the knee joint. The rats were divided in to three groups. One laser group had laser irradiation with power intensity 3.9 W/cm^2, another laser group had laser irradiation with power intensity 5.8 W/cm^2. The last group was treated with a sham laser. After 6 times of treatment over 2 weeks, the hind legs were prepared for indentation of the articular surface of the knee and for dual energy x-ray absorptiometry of the focused regions. The results showed that laser irradiation had potential for the prevention of biomechanical changes by immobilisation. Another study was on the effect of the laser irradiation on viscoelasticity of the contracted knee joint in rats (Usuba *et al.*, 1998). Forty-eight Wistar star rats were operated on to immobilise a knee joint. One week later, they were randomly assigned to four treatment groups. Two laser groups consisted of laser irradiations with $3.9W/cm^2$ and 40mW in output and with $5.8W/cm^2$ and 60mW in output. The third group was treated with whirlpool (swimming exercise) of 42°C. The last group was treated with a sham laser. Two weeks later, the hind legs were prepared for assessment of degree of knee contracture. The authors found that laser irradiation did not provide a significant effect in minimising the degree of experimental joint contracture over whirlpool treatment.

The effect of laser pulse repetition rate and pulse duration on mast cell numbers and degranulation was studied in Wistar rats (El Sayed & Dyson 1996). A $1cm^2$ partial thickness wound was made on the right flanks of 30 rats. The rats were divided into 5 groups. The centre part of the wound in the laser groups was treated with laser (wavelength: 820nm, time: 27 seconds, average power intensity: $800mW/cm^2$ and average power intensity: $21.6J/cm^2$) with different pulsing frequencies from 2.5 to 20,000 Hz. The controls received a sham laser treatment. All the rats were sacrificed after 2 hours. The wounds were removed, together with the underlying panniculus carnosus, fixed, and processed. Mast cells were stained and the number of intact and degranulated cells were counted. The authors found that laser irradiation in all frequencies investigated did not increase the total

number of mast cells. However, the 20 and 292 Hz frequency irradiation increased the ratio of degranulated mast cells.

Khullar *et al.,* (1995) studied the effects of LLLT on recovery of nerve conduction and motor function after compression injury in the rat sciatic nerve. Twenty rats received a standardised injury to the right sciatic nerve using a time, load and length sequence. GaAlAs laser irradiation (wavelength: 830nm, energy level: 6J), was applied to the skin of ten rats on the dorsal aspect of the hind leg overlying the site of the axonotmesis injury to the sciatic nerve. The sites were irradiated daily for 28 days. Ten rats remained as control. Nerve function was assessed by the "Sciatic Functional Index" involving evoked compound action potentials. At 21 days post injury, the laser–irradiated group had a significantly lower median "Sciatic Functional Index" than the controls. It was indicated that laser irradiation improved the functional recovery in the nerve. However, no differences were found between the evoked compound action potential parameters that were measured in the irradiated and control groups. Histological examination revealed the lack of difference between the two groups. The authors concluded that the effects of LLLT on recovery must have occurred more peripherally to the point measured.

1.9.1.2 Hard tissue healing

Bone healing has been reported to be enhanced following low level laser therapy. Takeda (1988) investigated the effect of LLLT on alveolar bone after tooth extraction. Maxillary right first molars of 24 - 8 week Wistar rats were extracted. The sockets of irradiated groups were irradiated with GaAs laser (Wavelength: 904nm, power intensity: 25mW/cm^2, energy intensity: 20 J/cm^2) for 5 minutes and subsequently, for 5 minutes daily. The irradiated and control groups were sacrificed at 0, 2, 4 and 7 days. One to 3 days before sacrifice, 1% lead disodium EDTA was injected for ossification labelling. The mesial wall of the socket was used for histopathological examination. The post-extraction healing was evaluated semi-quantitively on an arbitrary scale as absent, slight, mild and prominent. The authors found that the formation of trabecular osteoid tissue or newly formed bone, and the deposition of lead in newly formed trabecular bone appeared sooner

in the irradiated bone. Trelles & Mayayo (1987) also confirmed the bone healing enhancement by laser irradiation in mice. After applying He-Ne laser (wavelength: 632nm, energy level: 2.4J) to induced tibia fractures in mice, for 12 times, an increase in vascularisation and faster formation of osseous tissue with a dense trabecular net after about 4 weeks.

Saito & Shimizu (1997) described the effects of LLLT on bone regeneration during midpalatal suture expansion in rats. A total of 56 Wistar rats were used for the experiment. The rats were divided into 7 groups of 8 rats each. The first group had no treatment. The second group had the midpalatal suture expanded but no laser irradiation. The third to seventh groups had the suture expanded and different protocols for laser irradiation. The laser was a GaAlAs diode laser device (wavelength: 830nm, output: 100, power density: 35.3W/cm^2). All rats were triple bone labelled with calcein on day 0 and 6, and oxytetracycline on day 3. The rats were sacrificed on day 7. The maxillae were prepared for analysis. Another 20 rats were used for histological studies with similar protocol. It was found that the width between each labelling line was wider in the laser irradiation groups. The increase occurred in an irradiation dose-dependent and time-dependent manner. The authors concluded that LLLT significantly stimulated bone regeneration in the midpalatal suture during rapid maxillary expansion in rats.

1.9.2 Clinical studies

Although the animal studies of LLLT have produced promising results, some of the double blind clinical trials have had mixed outcomes. Khullar *et al.,* (1996) in a double blind study described the effect of LLLT on neurosensory deficits subsequent to sagittal split ramus osteotomy. Thirteen patients who had reduced sensibility after their final 2 –year post sagittal splint ramus osteotomy, were divided into two groups. The irradiated group (8 patients) received GaAlAs laser irradiation (wavelength: 820nm, energy level: 6J each site) on four sites along the distribution of the inferior alveolar nerve. The treatment consisted of 20 visits. The other 5 patients received equivalent placebo treatment. The degree of objective mechanoreceptor neurosensory deficit, thermoreceptor neurosensory deficit, and subjective neurosensory deficit was assessed. It was found that the real

irradiated group experienced a subjective improvement in both lip and chin after completion of the course of treatment. Moreover, there was a significant decrease in the area of mechanoreceptor neurosensory deficit in the real irradiated group. Neither the real irradiated nor control group had demonstrated any significant change or tendency to improvement in thermoperception on the completion of the course of treatment. The authors concluded that laser irradiation resulted in both a subjective and objective improvement in mechanical sensory perception in the long-standing neurosensory deficit of the inferior alveolar nerve. The study was well constructed and designed. The three different areas of assessment strengthened the validity of the findings.

While there may be accumulating evidence to support the analgesic capabilities of LLLT when used post-surgically, the mechanisms of this effect are unclear (Walsh 1997a). Double blind clinical trials were carried out to investigate the analgesic effect of LLLT. Heussler *et al.,* (1993) studied 25 women with active small joint rheumatic arthritis and treated them with GaAlAs laser (Wavelength: 839nm, energy intensity: 12 J/cm^2). The metacarpo-phalangeal and proximal interphalangeal joints of one hand of the patients were irradiated for 30 seconds. The other hand received a sham laser treatment. Each patient received 12 treatments over 4 weeks. Pain was assessed by visual analogue scale. Other objective assessments such as stiffness of joints, drug use, bone scan, haematological and serological tests were included. A total of 72% of patients reported pain relief but the reduction was reported equally in both hands. No other changes were seen in other clinical, functional, scintigraphic, or laboratory features. The authors concluded that LLLT had no objective effect on patients with rheumatoid arthritis. The authors suggested that LLLT appeared to produce analgesia through a powerful placebo effect.

The effects of pain reduction during orthodontic treatment have been investigated by double blind studies. Lim *et al.,* (1995) assessed the efficacy of LLLT in reducing pain caused by elastomeric separators. The same type of laser unit as the present study was used ie. P-LaserSystem. Thirty-nine dental students were chosen for the study. Separators were placed at the mesial and distal proximal contacts of

one premolar in each quadrant of the dentition. Pretreatment visual analogue scale scores were recorded 5 minutes after the placement of separators. The laser (wavelength: 830nm, output: 30mw, power intensity: 59.7mW/cm^2) was then applied to the buccal mucosa of the premolars for 15, 30 and 60 seconds treatment. A placebo application lasted 30 seconds. The post treatment scores for each premolar were recorded immediately after the laser exposure. The separators were left in situ for 5 days. The subjects recorded the scores again on the 2nd, 3rd, 4th and 5th days. The authors concluded that LLLT was unable to provide immediate pain relief. However, it could have potential in reducing the intensity of pain. Harazaki & Isshiki (1997) studies the effects of LLLT on pain reduction just after patients received the edgewise appliance with 0.012 to 0.016 inches nickel-titanium wires. He-Ne laser (wavelength: 632.8nm, output: 60mW) was applied to each tooth for 30 seconds from lingual and buccal surfaces of 20 patients. The blind group (20 patients) received a sham laser treatment. Another 44 patients served as controls. The assessment of pain was through questionnaire. It was found that all patients had pain but laser irradiation delayed the starting of pain by a few hours. Although there were more patients from the irradiated group that had milder pain, 50% of the irradiated patients were still in the most severe pain category. The blind and control groups had 65% and 52% of the most severe pain, and that of irradiated group was 50%. However, in clinical terms, the differences were only 1 to 3 patients. There were no differences between irradiated and blind groups on the day when the pain disappeared after application. Not surprisingly, the younger patients had lower pain sensitivity in all groups. Although the authors concluded that LLLT reduced the pain in some patients undergoing orthodontic treatment, it was the author's opinion that the pain reduction might not be clinically significant. It appeared that the only effect of LLLT was the delay of pain starting for a few hours.

Harazaki *et al.,* (1998) described the pain reduction effect by LLLT in orthodontic treatment. The same laser unit was used as the previous study. The experiment was a double blind study. The subjects were 34 patients (11 males, 23 females) treated with edgewise appliances. However, the sample size became 40 without explanation. Two groups of 20 patients were included in the study. In the laser

group, each painful tooth was irradiated with a laser for 3 minutes. The controls had treatment with composite curing light. The degree of pain was recorded as with the previous study. However, the authors stated that in the laser group, each painful tooth was irradiated labially and lingually for 30 seconds interval until effects were observed. The authors might be describing a second study but there is no clear indication. Electrical pulpal diagnosis was made before the application of the appliance, on the next day, and after laser irradiation. The authors concluded that they succeeded in reducing severe tooth pain due to orthodontic treatment by LLLT. However, the validity of the method and data was questionable. Hall & Freer (1998) suggested that the physiological changes in the pulp during orthodontic movement could alter the integrity of the A fibres. Response thresholds to electrical stimulation would increase and consequently the electric pulp test might not initiate a response at all. The use of electrical pulp testing to assess LLLT effects on pain in orthodontic treatment is inappropriate. Moreover, from the data presented in the article, the conclusion drawn by Harazaki *et al.*, (1998) is unsupported by evidence.

There were several studies about the effects of LLLT on pain reduction in dental surgical procedures. Fernando *et al.,* (1993) and Roynesdal *et al.,* (1993) conducted double blind studies to investigate the effects of diode laser on postoperative pain and swelling in patients after bilateral removal of mandibular third molars. Visual analogue and clinical signs were used to assess the treatment effects. Both groups of authors concluded that LLLT had no beneficial effect on pain or swelling.

Table 1.6 summarises the current studies on LLLT. The vast majority of published work finds clear evidence that laser irradiations alter cellular process in an athermal, wavelength, dose dependent manner. Standards have improved markedly over the last decade. However, current work remains more variable than might be desired. Evaluation of most trials is complicated by the waxing and waning of the conditions studied, frequent reliance on clinical diagnosis, and the semiquantitative nature of many treatment outcomes such as lessening of pain and stiffness (Basford 1995). Although the results of *in-vitro* studies showed that

LLLT had multiple therapeutic effects, the data from double-blind clinical trials showed less promising results (Tab. 1.6). In most of the clinical trials, the details of the treatment parameters eg. energy level and energy intensity, were not stated. It makes evaluation and comparison of the results difficult.

Table 1.6. Summary of current research

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Human foreskin fibroblasts	Cell culture	HeNe (633 nm) Output: 0.9 mW Power intensity: 24.7 mW/cm^2 Energy intensity: 1.48 J/cm^2 Time: 60 s daily for 5 days	No effect on proliferation	Hallman *et al.*, (1988)
Mouse spleen cells	Cell culture	He-Ne (632.8nm) Output: 0.68 mW/cm^2 Energy intensity: 0.0034 – 0.034 J/cm^2 Time: 5 – 50s; single exposure	180-250% of increased spontaneous chemiluminescence and candida-induced chemiluminescence Induced respiratory burst The effects was dose related	Karu *et al.*, (1989)
Human gingival fibroblasts	Cell culture	HeNe (632.8 nm) Output: not stated Power intensity: 10 mW/cm^2 Energy intensity: 1.2 J/cm^2 Time: 10 min each exposure; 1-3 exposures in 11 days	Single treatment resulted in 2 fold increased in cell number. Multiple exposures showed new increases Laser treatment induced mitochondrial hyperplasia and hypertrophy, morphologic changes at the ultrastructural level.	P-Schneider *et al.*, (1989)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Human skin fibroblasts	Cell culture	GaAlAs (904 nm) Peak output: 2 W Energy intensity: 0-20 mJ/cm^2 Pulse: 50-200 Hz Time: multiple exposures for 4 days	Increased ascorbic acid uptake Increased hydroxyproline formation	Labbe *et al.,* (1990)
Human gingival fibroblasts	Cell culture	HeNe (632.8 nm) Output: 10 mW Energy intensity: 1.2 J/cm^2 Time: 10 min single exposure	Rapid generation of myofibroblasts from fibroblasts in as early as 24 hours.	P-Schneider *et al.,* (1990)
NIH/3T3 fibroblasts	Cell culture	HeNe (632 nm) Output: 10 & 35 mW Diode (708 nm) Output: not stated Black light source (360nm) Power intensity: 2 mW/cm^2 Energy intensity: 0.1 - 90 J/cm^2 Time: Single exposure	Maximum mitosis at different energy dose for different wavelength. Mitosis decreased with higher doses in all wavelength	Lubart *et al.,* (1992a)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Human skin fibroblasts	Cell culture	HeNe (632.8 nm) Output: 10 mW Energy intensity: not stated Time: 2-20s 4 times in 48 hours	No increase in no of fibroblasts. Increased ATP production	Rigau *et al.,* (1991)
Mouse calvaria osteoblastic cells	Cell culture	He-Ne (632.8 nm) Output: 8.5mW Energy intensity: 0.01-1 J/cm^2 Time: 3.3 – 300s; single dose or daily for 4 days	Dose dependent increased of DNA synthesis No significant increase of alkaline phosphatase (ALP) activities Increase in Ca accumulation with 1 J/cm2 Enlargement of Golgi apparatus No elevation of temperature by irradiation	Yamada (1991)
Bull Sperm cells	Cell culture	HeNe (632 nm) Output: 10 & 35 mW Diode (780 nm) Output: 13 & 40 mW Energy intensity: 2-30 J/cm^2 Time: not clearly stated	Increased Ca^{2+} at low energy doses, decreased after higher doses	Lubart *et al.,* (1992b)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Quail embryo heart fibroblasts	Cell culture in 3D hydrated collagen lattice	HeNe (632.8 nm) Output: not stated Energy dose: 1.5 J/cm^2 Time: 2 mins. single exposure	Decreased speed and increased frequency and duration of stops	Noble *et al.,* (1992)
Human oral mucosa fibroblasts	Cell culture	GaAlAs (812 nm) Output: 84 mW Power intensity: 4.5 mW/cm^2 Energy intensity: 0.0045 – 4.5 J/cm^2 Time: 1- 1000s single exposure	Increased DNA synthesis at 0.45 J/cm^2	Loevschall & Arenholt-Bindslev (1994)
HeLa cells	Cell culture	He-Ne (623.8 nm) Power intensity: 1 mW/cm^2 after filtered Energy intensity: 0.001 J/cm^2 Time: 10s; single exposure at 5, 60 or 180 mins before gamma irradiation	With laser irradiation 60 or 180 minutes before gamma irradiation, the repair of gamma-irradiation damage was speeded up	Karu *et al.,* (1994b)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Human embryonic fibroblasts	Cell culture	GaAs (904nm) Output: 2.5 – 4.2 mW Energy intensity: 0.077 – 1.044 J/cm^2 Pulse: Constant but not stated Frequency: Constant but not stated Time: 10 – 80s; single exposure or twice daily for 4 days	Single or twice laser irradiations had no effects on collagen synthesis Increased procollagen synthesis after multiple irradiations	Skinner *et al.*, (1996)
Guinea pig skin wound	Animal	Ar (wavelength not stated) Power intensity: 20 mW/cm^2 Energy intensity: 2 J/cm^2 Time: 100s; exposures every 2 to 3 days	No effect on wound healing	McCaughan *et al.*, (1985)
Fractured tibia of white mice	Animal	He-Ne (632 nm) Output: 4 mW Energy level: 2.4 J Energy intensity: not stated Time: 10 mins; every second day, 1 - 12 exposures	Increased in vascularisation and faster formation of osseous tissue with a dense trabecular network	Trelles & Mayayo (1987)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Rat extraction wound	Animal	GaAlAs (904 nm) Power intensity: 25 mW/cm^2 Energy intensity: 20 J/cm^2 Time: 5 mins. daily for 7 days	More prominent proliferation of fibroblasts and formation of trabecular osteoid tissue A more rapid ossification in treatment group	Takeda (1988)
Wistar Rats Thyroid glands	Animal	GaAs (904 nm) Peak output: 50 W Energy level: 10-60J Energy intensity: not stated Time: 2-15 days	Morphological and molecular changes of thyroid cells	Hernandez *et al.*, (1989)
Infected rat skin wound	Animal	GaAs (904 nm) Average Output: 2 mW Pulse rate: 1000 Hz Energy intensity: 76.4 J/cm^2 Time: 2 mins. exposures on 1st, 3rd, 5th day	Increased rate of wound closure Decreased incidence swelling	Lee *et al.*, (1993)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Dorsal aspect of hind leg overlaying damaged sciatic nerve in rat	Animal	GaAlAs (830 nm) Energy level: 6 J Energy intensity: not stated Time: Daily for 28 days	Improved functional recovery in the nerve No effects on evoked compound action potentials No histological differences	Khullar *et al.*, (1995)
Wistar rats skin wound	Animal	GaAlAs (820 nm) Output: 100 mW Power intensity: 800 mw/cm^2 Energy intensity: 21.6 J/cm^2 Pulse: 2.5-20000 Hz Pulse duration: 360-0.045 ms Time: 27 s; single exposure	Increased number of mast cells in all frequencies Increased number of degranulated mast cells in 20 and 292 Hz groups only Degranulation is pulsing frequency dependent	El Sayed *et al.*, (1996)
Dairy Cattle Teats wound	Animal	HeNe (632.8 nm) Output: 8.5 mW Energy intensity: 3.64 J/cm^2 Time: single exposure	Accelerated wound healing	Ghamsari *et al.*, (1996) Ghamsari *et al.*, (1997)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Immobilised Wistar rat knee joint	Animal	GaAlAs (810nm) Output: 40 & 60 mW Spot size: 1.04 mm^2 Power intensity: 3.9 & 5.8 W/cm^2 Energy level: 7.2 & 10.8 J Energy intensity: 702 & 1044 J/cm^2 Time: 3 mins; 6 exposures in 2 weeks	Preservation of articular cartilage stiffness Prevention of biomechanical changes by immobilisation	Akai *et al.,* (1997)
Midpalatal suture of rat	Animal	GaAlAs (830 nm) Output: 100 mW Power intensity: 35.3 W/cm^2 Energy level: 18 – 126 J Energy intensity: 6390 – 44730 J/cm^2 Total energy level: 126 & 420 J Time: 3 or 10 mins; daily for 7 days or 7 minutes; 3 exposures at different time or 21 minutes; single exposure	Accelerated bone regeneration in a midpalatal suture during rapid palatal expansion The effects were dose, timing and frequency dependent	Saito &Shimizu (1997)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Immobilised Wistar rats knee joint	Animal	GaAlAs (810nm) Output: 40 & 60 mW Spot size: 1.04 mm^2 Power intensity: 3.9 & 5.8 W/cm^2 Energy level: 7.2 & 10.8 J Energy intensity: 702 & 1044 J/cm^2 Time: 3 mins; 6 exposures in 2 weeks	No obvious advantages in minimising joint contracture compared to the conservative whirlpool method	Usuba *et al.*, (1998)
Six points over incisions after surgical removal of mandibular third molars	Double-blind clinical trial	He-Ne (623.8 nm) Power intensity: 0.3 W/cm^2 Energy intensity: 10 J/cm^2 Spot size: 1.8 mm^2 Time: not stated. Single treatment for 6 points	Reduced in trismus (same as ibuprofen group) No reduction in pain No reduction in swelling	Carrillo *et al.*, (1990)
Human gingival wound	Clinical trial	HeNe (632.8 nm) Output: 10 mW Energy intensity: not stated Time: 2 min single exposure	Myofibroblasts in connective tissue 48 hours after LLLT. No myofibroblasts in untreated site.	P-Schneider *et al.*, (1990)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Wounds after surgical removal of mandibular third molars	Clinical trial	HeNe (632.8 nm) Pulse: 50 Hz Output: 8 mW Energy intensity: not stated Time: 2 mins; single exposure	No difference in postoperative swelling and pain *Study was discontinued for ethical reasons	Taube *et al.,* (1990)
Sockets after surgical removal of mandibular third molars	Double-blind clinical trial	Semi-conductor (830 nm) Output: 30 mW Energy level: 4 J Energy intensity: not stated Time: 132s; sweeping movement; single exposure	No effects on pain and swelling reduction	Fernando *et al.,* (1993)
Active rheumatoid arthritis sufferers	Double-blind clinical trial	GaAlAs (830 nm) Output: not stated Energy intensity: 12 J/cm^2 Time: 30 s; 12 exposures in 4 weeks	No objective effect on patients with rheumatoid arthritis. Powerful placebo analgesia effect.	Heussler *et al.,* (1993)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Mandible after third molars extraction	Double-blind clinical trial	GaAlAs (830 nm) Output: 40 mW Energy level: 6 J Energy intensity: not stated Time: pre and post operation	No beneficial effect on swelling, trismus, and pain after third molar surgery	Roynesdal *et al.*, (1993)
Tooth after orthodontic separation	Double-blind Clinical trial	GaAlAs (830 nm) Output: 30 mW Power intensity: 59.7 mW/cm^2 Energy intensity: 9 - 35.8 J/cm^2 Time: 15-60s; daily for 5 days	No differences on the levels of pain	Lim *et al.*, (1995)
Four sites along ID nerves with reduced sensibility after osteotomy	Double-blind clinical trial	GaAlAs (820 nm) Energy level: 6 J Energy intensity: not stated Time: 4 sites per session; total 20 sessions	Improved both subjective and objective mechanical sensory perception in long-standing neurosensory deficit in the ID nerve No improvement on thermoception	Khullar *et al.*, (1996)

Target Tissue	Model	Laser Treatment Parameters	Reported Effect	Researcher
Teeth undergoing orthodontic treatment	Double-blind clinical trial	He-Ne (623.8nm) Output: 6mW Energy intensity: not stated Time: 30s on lingual and labial surfaces. 12 - 24 minutes per patient; single exposure	Delay in pain appearance. Reduction in pain in some patients	Harazaki & Isshiki (1997)
Painful teeth after orthodontic adjustment	Double-blind clinical trial	He-Ne (628.8nm) Output: 6mW Energy intensity: not stated Time: Multiple of 30s on lingual and labial surfaces until effects were observed (2 - 5 minutes per tooth)	Pain reduction in 112 of 114 teeth as measured by electrical pulp test	Harazaki *et al.*, (1998)

1.10 Absorption of laser energy in LLLT

Proteins appear to be the major absorbing structures for the red visible and near infra-red laser wavelengths used in LLLT. However, the identity of the photoreceptors responsible for the biological effects of LLLT has not been resolved (Walsh 1997a). Wharton and Cusanovich (1969) adduced evidence that absorption of photons was largely due to cupric copper in enzymes. Wharton and Gibson (1968) drew attention to a relatively weak absorption band of cytochrome oxidase in the near infrared (800 - 900 nm). More recently, the near-infrared absorption of cytochrome oxidase has been used as an independent check on the valence state of components of the enzyme under different experimental conditions (Powers *et al.,* 1979). Near-infrared spectroscopy has also been used to measure cytochrome oxidase Cu_A reduction-oxidation (redox) state in haemoglobin deoxygenation (Cooper & Springett 1997). Friedmann *et al.,* (1991) described a possible explanation of laser-induced stimulation and damage of cell cultures. They proposed that the cytochrome oxidase in mitochondria and low level of porphyrin present in the cytosome might account for the absorption of infrared diode-laser light in the region between 700 and 900 nm.

1.11 Primary response

The primary mechanisms of light action on the photoreceptor molecules have not yet been established (Walsh 1997a). Here a few possibilities discussed in the literature so far are considered.

1.11.1 Changes in redox properties

It has been suggested that one of the possible processes involves an acceleration of electron transfer in the respiratory chain due to a change in the redox properties of the carriers following photoexcitation of their electronic states (Friedmann *et al.,* 1991). Electron excitation changes the redox properties of molecules. Karu (1998) proposed that the photoexcitation of certain reaction centres in the cytochrome oxidase molecule (like Cu_A and Cu_B) influenced the redox state of these centres and consequently the rate of electron flow in the molecule. It was also suggested

that irradiation shifted the cytochromes to more reduced forms which naturally promoted electron transport.

1.11.2 Local transient heating of chromophores

During light excitation of electronic states, a noticeable fraction of the excitation energy is inevitably converted to heat, which causes a local increase in the temperature of the absorbing chromophores (Karu 1998). Any appreciable average heating of the targeted tissue can be prevented by controlling the irradiation intensity and dose appropriately. However, there is still the possibility of localised transient heating of absorbing chromophores. The local transient rise in temperature of absorbing biomolecules may cause structural changes and trigger biochemical activity such as activation or inhibition of enzymes (Karu 1998). The minimum size of the microregions transiently heated by irradiation with fentosecond laser pulses is estimated at about 10 Å which corresponds to the size of the chromophores of the hypothetical primary photoreceptors – the respiratory chain components (Karu 1998).

The effects of slight local heating and a substantial local temperature gradient occur for both continuous wave and fentosecond pulses. The difference is that the density of such local heating sites in the case of continuous wave radiation is extremely low because of the low radiation intensity. In the case of fentosecond pulses, the chromophores become excited, which causes a stronger biological response (Karu 1998).

1.11.3 One electron auto-oxidation

It has been shown that in mitochondrial electron transport, superoxide radicals are produced (Karu 1998). The production of O_2^- as well as the product of its dismutation, H_2O_2, primarily depends on the metabolic state of the mitochondria. By activating electron flow in the respiratory chain, O_2^- production is expected to increase (Karu 1998).

It has been demonstrated that mitochondria possess a mechanism for the resorption of O_2^-, and O_2^- may be a source of electrons for the oxidative

phosphorylation of ADP under physiological conditions (Karu 1998). Even a small increase in O_2^- concentration in a cell may result in multiple responses like the increase in intracellular [Ca^{2+}] and pH (alkalisation), release of arachidonate, activation of Na^+/H^+ antiport and Ca^{2+} ATPase, and alternation of Na^+ - Ca^{2+} exchange (Friedmann *et al.,* 1991).

Karu (1998) suggested that some low level laser effects could be related to an increase in O_2^- production. Moreover, H_2O_2 is involved in a complex set of reactions which link the H_2O_2 production in mitochondria to the regulation of cellular metabolism. Many antioxidant enzymes are involved in this link. One of the enzymes taking part in the pathway is catalase. Catalase is shown to be involved in the increase in protein synthesis induced by He-Ne laser light in yeast cells (Karu 1998).

1.11.4 Photodynamic action

Certain photoabsorbing molecules like porphyrins and flavoproteins can be reversibly converted to photosensitisers. The absorption of light quanta by these molecules is responsible for the generation of singlet oxygen, $^1O_2$. Oxygen at chemically active states with energies of 1.0 and 1.5 eV may play an active mediator role in achieving the biological effects of irradiation. The singlet oxygen is a potent oxidiser and stimulates the redox activity of the respiratory chain in mitochondria (Friedmann *et al.,* 1991). The light effects on respiration are oxygen-dependent and prevented by anaerobiosis. Karu (1998) proposed that photodynamic reactions promoted by certain respiratory chain components were associated and occur in aerobic conditions only.

Taken together, there is certainly more than one reaction involved in the primary mechanisms of low level laser effects. Karu (1998) reviewed the recent experimental results of measurements of redox absorbance changes of living cells after the irradiation. The results indicated that mechanism based on changes in redox properties of terminal enzymes of respiratory chains might be crucial (Karu 1998).

Today, LLLT is used world-wide including Australia and new uses are regularly reported but their effects and mode action are not fully determined. The use of LLLT in patient treatment should proceed carefully until objective evidence to support its use in the clinical conditions is reported. Although effects on cellular functions by LLLT including promotion and inhibition of protein synthesis, promotion and inhibition of cell growth and differentiation, increase in cell motility, improvement of membrane potential and binding affinities, increase in neurotransmitter release, increase in phagocytosis, increase in ATP and prostaglandin synthesis have been suggested, the mechanisms of the laser and tissue interaction have not been established (Basford 1995). Standards of research have improved markedly over the last decade, however, there is no standard protocol of LLLT in treating even the same medical condition. The effects of wavelength, beam type, power output, energy level, energy intensity, exposure regime of LLLT remain unexplained.

Animal studies of LLLT on bone healing showed favourable results (Trelles & Mayayo 1987, Takeda 1988, Saito & Shimizu 1997). Cell culture study of LLLT using mouse osteoblastic cells also showed an increase in DNA synthesis and enlargement of Golgi apparatus (Yamada 1991). However, there are no reported studies of LLLT on human osteoblastic cell lines.

1.12 Aims of the study

To investigate the effects of low level laser irradiation on the human osteosarcoma cell line SAOS-2

1.13 Objectives of the study

1. To describe the late effects of low level laser irradiation with different energy levels on cellular activities of human osteosarcoma cells
2. To describe the early effects of low level laser irradiation with different energy levels on cellular activities of human osteosarcoma cells

1.14 Hypothesis

HA: Low level laser irradiation has late effects on cellular activities

HB: Low level laser irradiation has early effects on cellular activities

1.15 Null Hypothesis

H_0: Low level laser irradiation has no effects on cellular activities

CHAPTER TWO

MATERIALS AND METHODS

2.1 Laser equipment

A continuous wave portable diode laser (P-LaserSystem, International Aps, Denmark) was used in the study. The laser consisted of three parts: a rechargeable battery pack (Fig. 2.1), a laser head (Fig. 2.2) and an optical tip (Fig. 2.3).

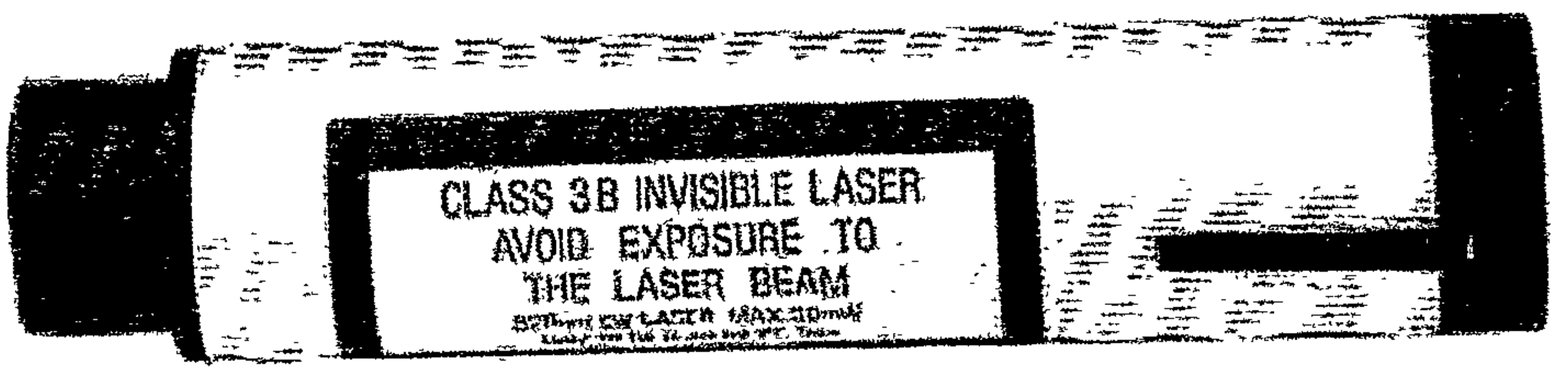

Figure 2.1. Rechargeable battery pack

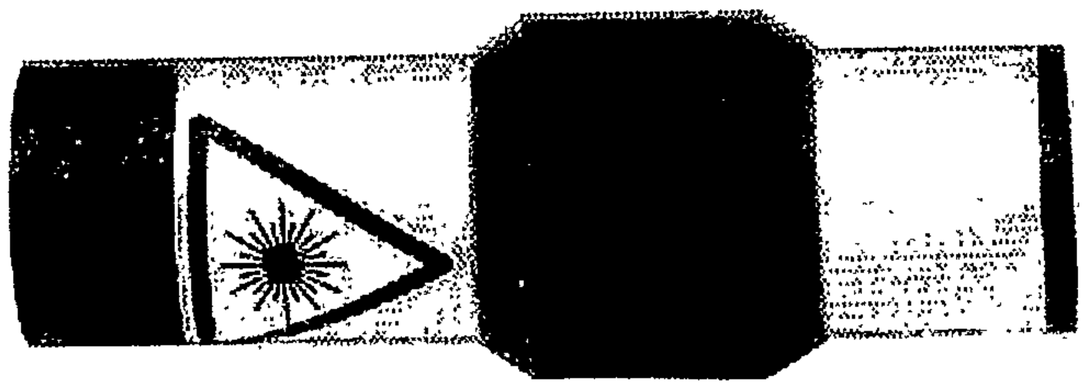

Figure 2.2. Laser Head

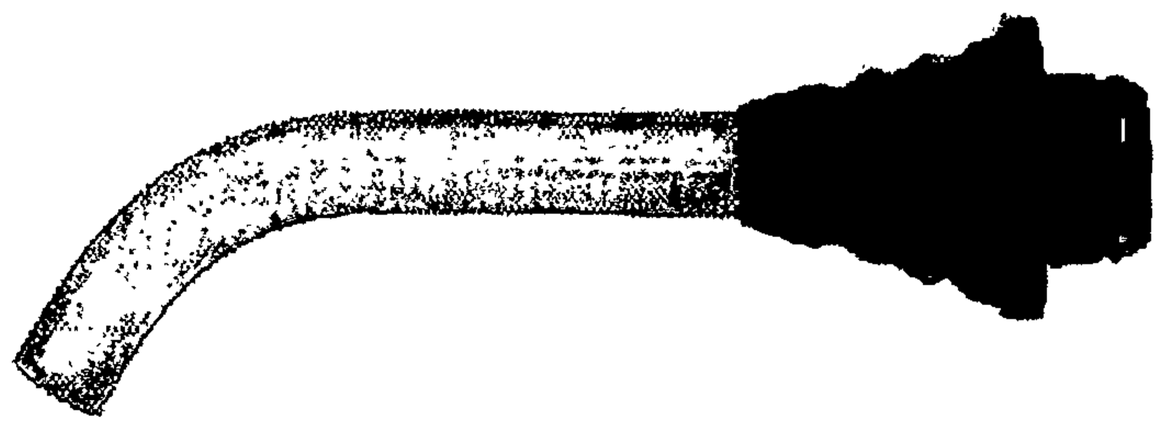

Figure 2.3. Optical tip

The different parts were assembled as shown in Figure 2.4. The laser was run on battery only. The rechargeable battery was charged overnight (12 hours for full recharge) at AC outlet (110 V) before used. Since the AC voltage was higher in Australia, a transformer was used to step down the AC voltage. The LED in the battery pack changed yellow to green when the battery had been recharged for 12 hours. However, the colour of the LED did not indicate the power status of the battery. If the battery was a fully recharged (after 12 hours and the LED had turned green) and then was plugged into the power point again, the LED would still be yellow.

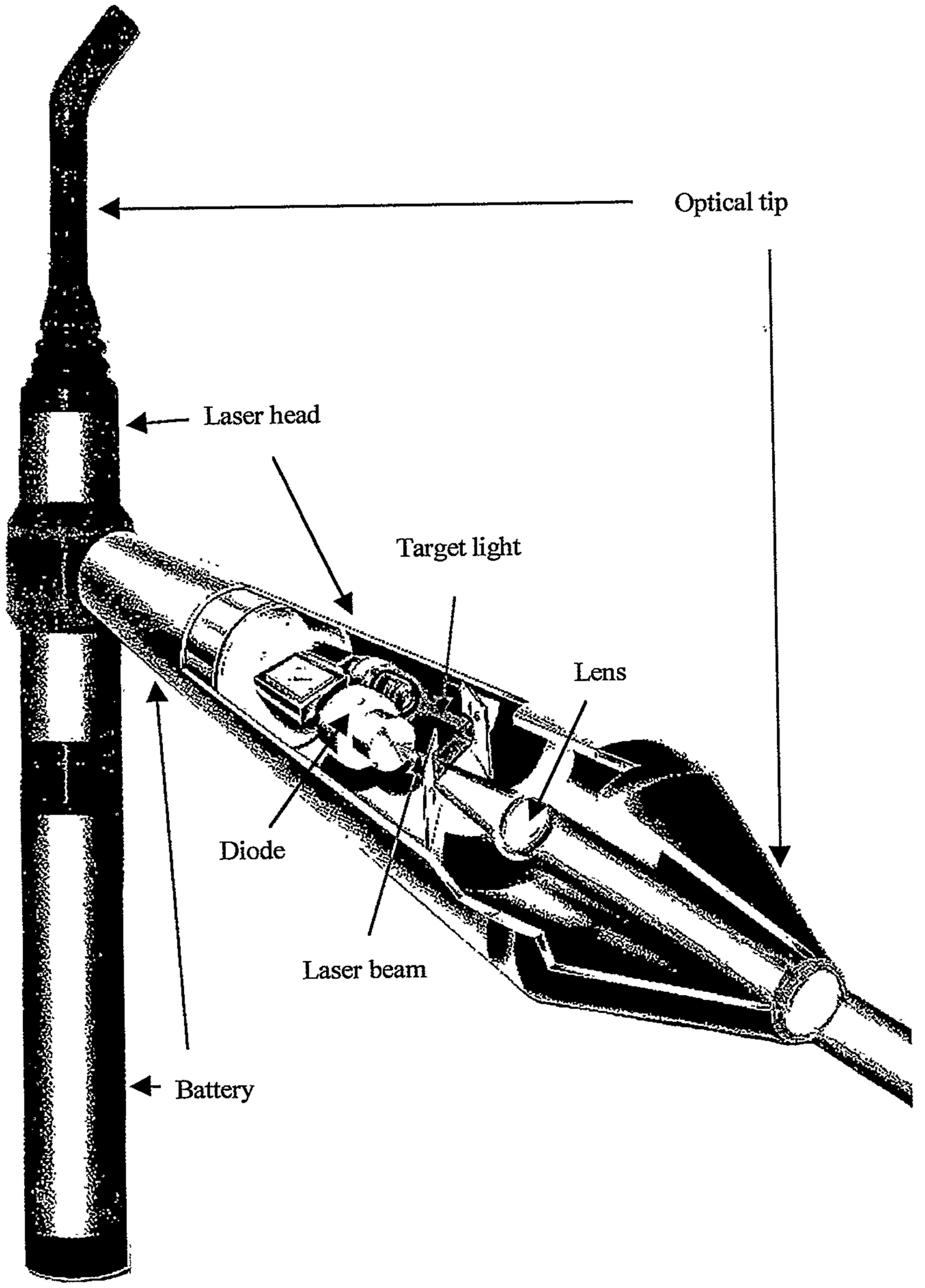

Figure 2.4. Structure of P-LaserSystem

Manufacturer specifications were listed as below.

Laser class:	3B
Laser medium:	Gallium-Alumnium-Arsenido diode (GaAlAs)
Wavelength:	830nm (invisible)
Energy Output:	70mW
Beam mode:	Continuous wave
Spot size:	4.5mm in diameter
Optical tip:	9mm in diameter (75-95% transmission rate)
Target light:	Visible red spectrum
Working time:	Minimum 100 minutes after a full recharge (12 hours)

Visible red light was emitted from the laser unit as the target light. The unit incorporated a timer which signalled every 0.5 second with a beeping sound. Although the diameter of the optical tip was 9mm, the spot size was at 4.5mm in diameter. A power meter which was provided with the unit was used to check the energy output during the experiment. (Fig. 2.5)

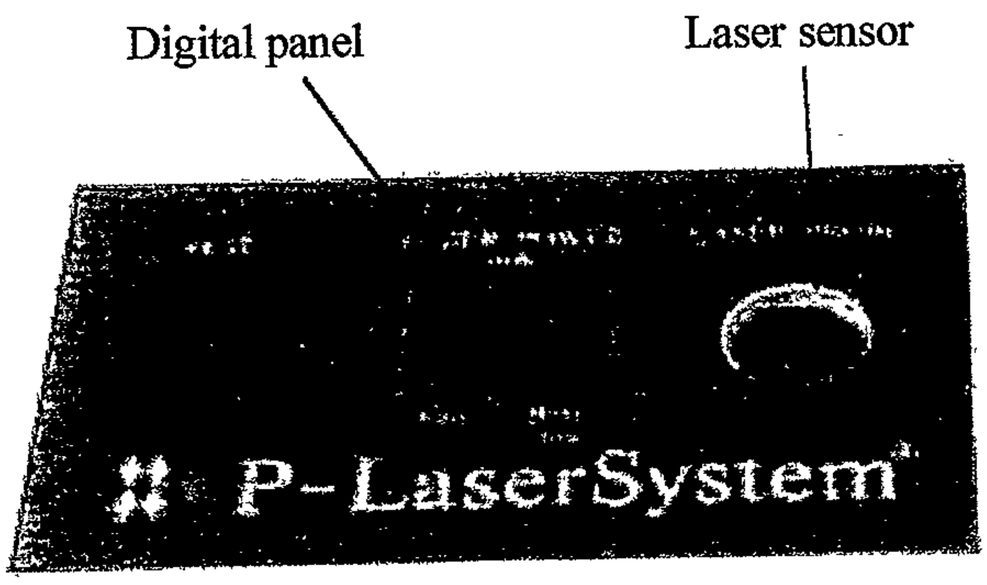

Figure 2.5. Power meter

2.2 Cell culture

SAOS-2 cells were obtained from the Department of Oral Pathology, Westmead Centre for Oral Health. This cell line was a human osteosarcoma cell line which was derived from an 11 year old caucasian female (Fogh *et al.*, 1977a, Fogh *et al.*, 1977b). These cells were immortal with a doubling time of 37 hours (Rodan *et al.*, 1987). This cell line possessed many osteoblastic properties including 1) formation of a calcifying matrix in diffusion chambers, 2) expression of very high levels of alkaline phosphatase (ALP), 3) presence of parathyroid hormone receptors coupled to adenylate cyclase, 4) presence of vitamin D receptors and 5) production of osteonectin (Rodan *et al.*, 1987). SAOS-2 were cultured in plastic culture flasks in Dulbecco's Modification of Eagle's Medium (DMEM, ICN BioMedical, Australia, Appendix 1) with 10% foetal calf serum and L-glutamine supplement (Appendix 2) at 37°C in a CO_2 incubator (5% CO_2, 95% air). The cells were passaged every 4 days using 0.1% trypsin (Sigma St. Louis USA) plus 0.02% EDTA in phosphate-buffer saline (PBS) (Oxoid Ltd. England).

2.3 Harvest and seeding

A confluent flask was rinsed with PBS twice. The cells were trypsinised and transferred to a 15mL tube. The cells were then centrifuged at 170g (relative centrifugal force, rcf) for 10 minutes at 4°C. The number of cells per mL were counted in a heamocytometer. The cells were then seeded into 96 wells plates at different seeding density with 200μL medium before irradiation. The depth of an individual well was 10.8mm. The internal diameter of the well was 6.4mm and the external diameter, measured at the rim, was about 9mm. The growth area was $0.32cm^2$ per well.

2.4 Irradiation procedure

A seeded plate was transferred from the incubator into the laminar-flow chamber. The lid of the plate was removed and the wells were laser irradiated with designated energy levels. The optical tip was placed on the rim of the well with the centre of the laser beam targeting to the centre of the well. The optical tip which had the same diameter of rim of the well fitted very well on the top the well. A black plastic board was placed at the bottom of the plate to minimise

scattered irradiation from reflection. The energy output was tested with the power meter at the beginning of the each experimental session and then every 10 minutes. The energy output was also tested with a plastic disc cut from the bottom of a well placed on the laser sensor in the meter. The energy output was tested again with a cut out well containing 200μL medium placed on the laser sensor in the meter.

The energy output specified by the manufacturer was 70mW. When the laser was tested on the power meter provided, the energy output was 78±2mW. The output decreased to 66mW with the plastic disc and to 58mW with a cut well containing 200μL culturing medium. Therefore, the actual laser energy reaching the bottom of the well where the cells grew was calculated to be 70mW. The timer in the battery pack signalled with a beep every half a second. The energy levels and intensities for the pilot study were set as follows.

Time of irradiation	Nearest energy level	Nearest energy intensity
4 seconds	0.28J	0.018J/mm^2 (1.8J/cm^2)
8 seconds	0.56J	0.035J/mm^2 (3.5J/cm^2)
15 seconds	1.05J	0.066J/mm^2 (6.6J/cm^2)
30 seconds	2.1J	0.132J/mm^2 (13.2J/cm^2)
60 seconds	4.2J	0.264J/mm^2 (26.4J/cm^2)

The battery cycle of the laser was stated as 100 minutes after being fully recharged. However, the actual laser operating time for continuous use was about 40 minutes after being fully recharged. It took 2 minutes to irradiate one set of wells with 5 different energy levels. In a 7-day experiment, the maximum sample size of each laser group was limited to four. The short operating time of the laser severely affected the sample size of the experiments.

The laser unit was broken after the pilot study. It was sent to Liberato-Lasers, Denmark for repair and the laser head was changed. The laser operating time of the repaired unit was further reduced to about 30 minutes. It had an energy output of 98mW when it was tested with the power meter. The output decreased to

86mW with the plastic disc and to 78mW with the cut well containing 200μL culturing medium placed on the top of the laser sensor. Therefore, the actual laser energy reaching the bottom of the well where the cells grew was calculated to be 90mW. The energy levels and intensities of the experiments were set to the integral of half a second as follows.

Time of irradiation	Nearest energy level	Nearest energy intensity
3 seconds	0.27J	0.017J/mm^2 (1.7J/cm^2)
5.5 seconds	0.50J	0.032J/mm^2 (3.2J/cm^2)
11 seconds	0.99J	0.062J/mm^2 (6.2J/cm^2)
22 seconds	1.98J	0.124J/mm^2 (12.4J/cm^2)
44 seconds	3.96J	0.249J/mm^2 (24.9J/cm^2)

In order to minimise the time when the plates were placed outside the incubator, each plate was irradiated for about 2½ minutes and then put back into the incubator. A second plate was then irradiated for 2½ minutes as the first one. The same procedure was repeated until all the plates were irradiated with the required regime. It took three cycles to finish irradiating one plate.

2.5 MTT Assay

This is an assay for measurement of mammalian cell survival and proliferation (Mosmann 1983). In living cells, mitochondrial succinate dehydrogenase reduced yellow tetrazolium salt (MTT, 3-[4,5 dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide) to blue formazan. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells (Mosmann 1983). The assay reflected the total culture biomass. The formazan formed was an insoluble blue salt but was soluble in ethanol and isopropanol.

A modified procedure from Mosmann (1983) was used:

1) Gently tip out the medium
2) Rinse the cells with 100μL PBS twice
3) Tip out PBS
4) Add 100μL serum free medium to each well

5) Add 10μL MTT 5mg/mL (Sigma USA, Appendix 3) to each well
6) Incubate for 3 hours at 37°C
7) Gently tip out MTT/medium
8) Add 100μL absolute ethanol to each well and mix with pipette
9) Read the result with multiwell spectrophotometer (Titertek Twinreader® Plus, EFLAB, Finland)
10) Measure the absorbances at 570nm (test wavelength) and 630nm (reference wavelength)

2.6 Alkaline phosphatase activity assay

Skeletal alkaline phosphatase (skeletal ALP) is a product of osteoblasts (Farley *et al.,* 1989). Skeletal alkaline phosphatase activities were shown to correlate with the rate of bone formation (Farley & Baylink 1986). Alkaline phosphatase activity assay was employed to measure the osteoblastic activities and hence the rate of bone formation. SAOS-2 was found to contain exclusively skeletal ALP and have high ALP activities (Farley *et al.,* 1989). Alkaline phosphatase converted phosphatase substrate (p-nitrophenyl phosphate disodium) to nitrophenol which was yellow in colour. The absorbances were measured at 405nm. The results were expressed as:

1) rate of absorbances at 405nm
2) nmol p-nitrophenol liberated per minute
3) stimulation ratio compared with control

In order to draw the relationship between absorbance and concentration of p-nitrophenol, standard curves with known concentration of p-nitrophenol were constructed. An effect of laser irradiation on ALP activities in SAOS-2 cell line might indicate an effect of laser irradiation on rate of bone formation.

Protocol 1:

1) Gently tip out medium
2) Rinse the cells twice with 100μL cold PBS
3) Tip out PBS (~4μL liquid left)
4) Add cold 54μL 0.1M glycine–NaOH buffer: pH 10.5 (Appendix 4) to experimental wells and sample blanks

5) Add 42μL PBS to substrate blanks
6) Wrap the plate with foil and store it at 4°C for 5 minutes
7) Place plate on ice tray
8) Add 42μL 5mg/mL phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma 104® USA) to experimental wells and sample blanks
9) Read the result with multiwell spectrophotometer (Titertek Twinreader® Plus, EFLAB Finland) immediately at 30°C
10) Measure the absorbances at 405nm every minute for at least 3 minutes

Protocol 2:

1) Gently tip out medium
2) Rinse the cells twice with warm 100μL PBS
3) Tip out PBS (~4μL left)
4) Add warm 54μL 0.1M glycine-NaOH buffer pH 10.5 to experimental wells and sample blanks
5) Add 42μL PBS to substrate blanks
6) Add 42μL 5mg/5mL phosphatase substrate to experimental wells and sample blanks (2 rows)
7) Incubate for 3 minutes at 37°C (2 minutes for day 5 plate)
8) Add 50μL NaOH 1M to stop reaction
9) Add 42μL 5mg/mL phosphatase substrate to remaining experimental wells and sample blanks (2 rows)
10) Incubate for 3 minutes at 37°C (2 minutes for day 5 plate)
11) Add 50μL NaOH 1M to stop reaction
12) Read the result with multiwell spectrophotometer (Titertek Twinreader® Plus, EFLAB Finland)
13) Measure the absorbances at 405nm

2.7 SDS Polyacrylamide gel electrophoresis: (SDS-PAGE)

Laser irradiation is reported to increase DNA synthesis (Loevschall *et al.,* 1994), increase hydroxyproline formation (Labbe *et al.,* 1990) and induce enlargement of the Golgi apparatus (Yamada 1991). In order to investigate the effects of laser

irradiation on the quantitative and qualitative changes of protein expression, proteins were extracted from cells after multiple laser irradiations and were loaded onto polyacrylamide gels (mini-gels). Proteins were separated by electrophoresis according to their molecular weights (Laemmli 1970). The proteins were stained with Coomassie blue and silver stain. The results of Coomassie blue staining were the quantitative (Laemmli 1970) and those of silver staining were qualitative (Gottlieb & Chavko 1987).

Procedure:

1. Prepare mini-gels with stacking gels (Mini-Protean II Electrophoresis Cell Instruction Manual)
2. Gently tip out medium
3. Rinse the cells twice with PBS
4. Add 100μL of PBS
5. Harvest cells by scraping the cells off the well
6. Transfer the cells to Eppendorf tubes
7. Centrifuge at 13,800g (rcf) for 2 minutes
8. Gently tip out suspension
9. Add 50μL running buffer (Appendix 5)
10. Add 25μL sample buffer (Appendix 5)
11. Pinch a hole on the lid of Eppendorf
12. Heat preparation on water bath for 5 minutes
13. Load mini-gels with preparation
14. Run the stacking gel at 60V
15. Run the gel at 120V
16. Remove gels after electrophoresis
17. Fix gels with fixer
18. Stain gels with Coomassie blue overnight
19. Destain with 5% acetic acid
20. Scan gels with flat top scanner
21. Stain gels with silver stain (Appendix 6)
22. Print black and white image of gels on transparencies and scan print out with Ultrascan XL (Laser Densitometer, LKB, Bromma)

2.8 Pilot Study:

The purposes of the pilot study were:

1. To determine the seeding density.
2. To determine the time window for observable changes.

2.8.1 Seeding density

Nine plates were seeded at 5,000, 10,000, 15,000, 20,000 cells per well (cells/well) as shown in Figure 2.6. MTT assay was performed on one plate from day 1 to day 9. The cells were fed on day 3 and day 6.

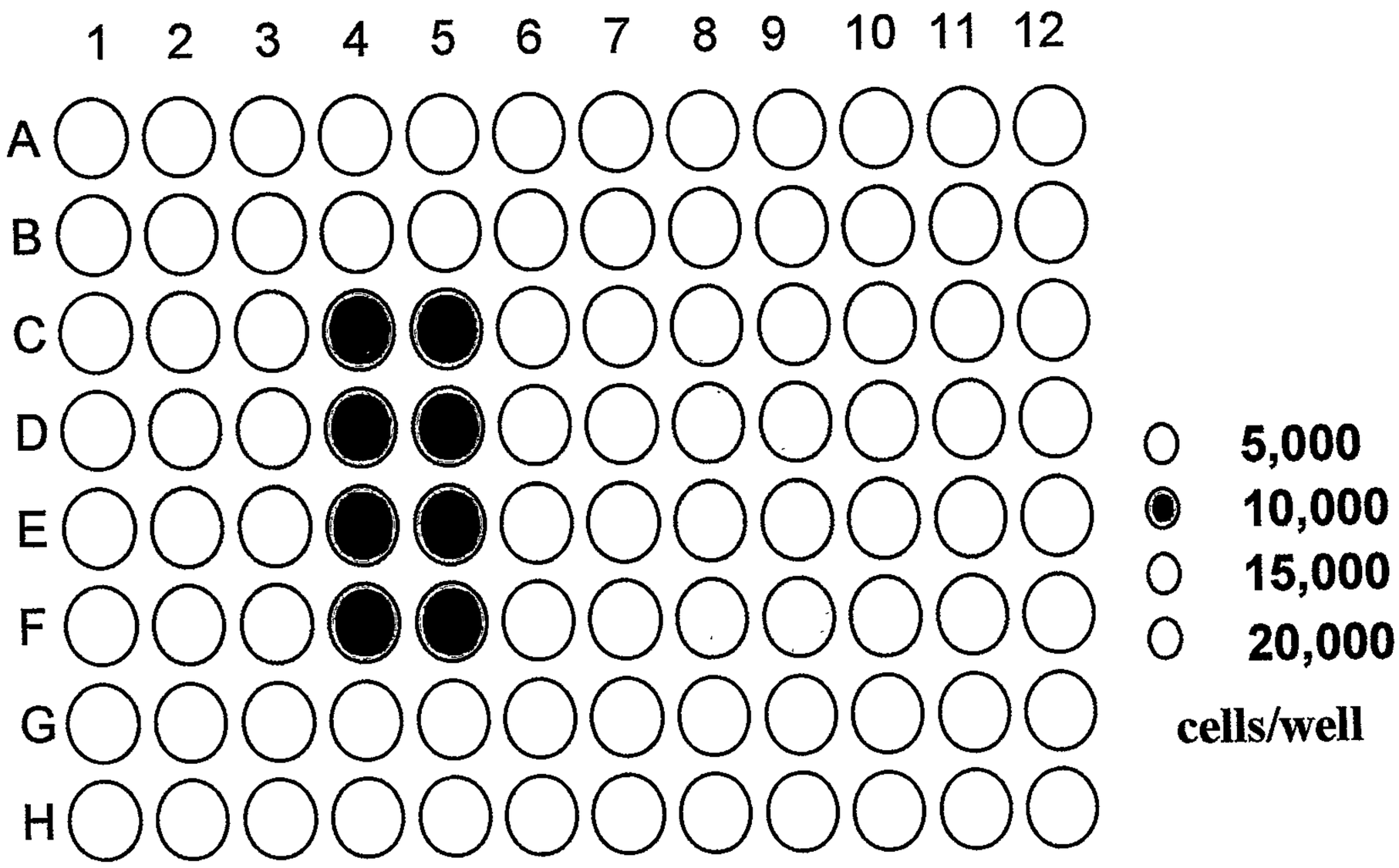

Figure 2.6. Well arrangement for pilot study with different seeding densities

2.8.2 Time window for observable changes

Six plates were seeded with 10,000 cells/well. The cells were irradiated 24 hours after seeding and daily subsequently as shown in Figure 2.7. MTT assay was performed 24 hours after irradiation. Wells located in the same columns served as controls.

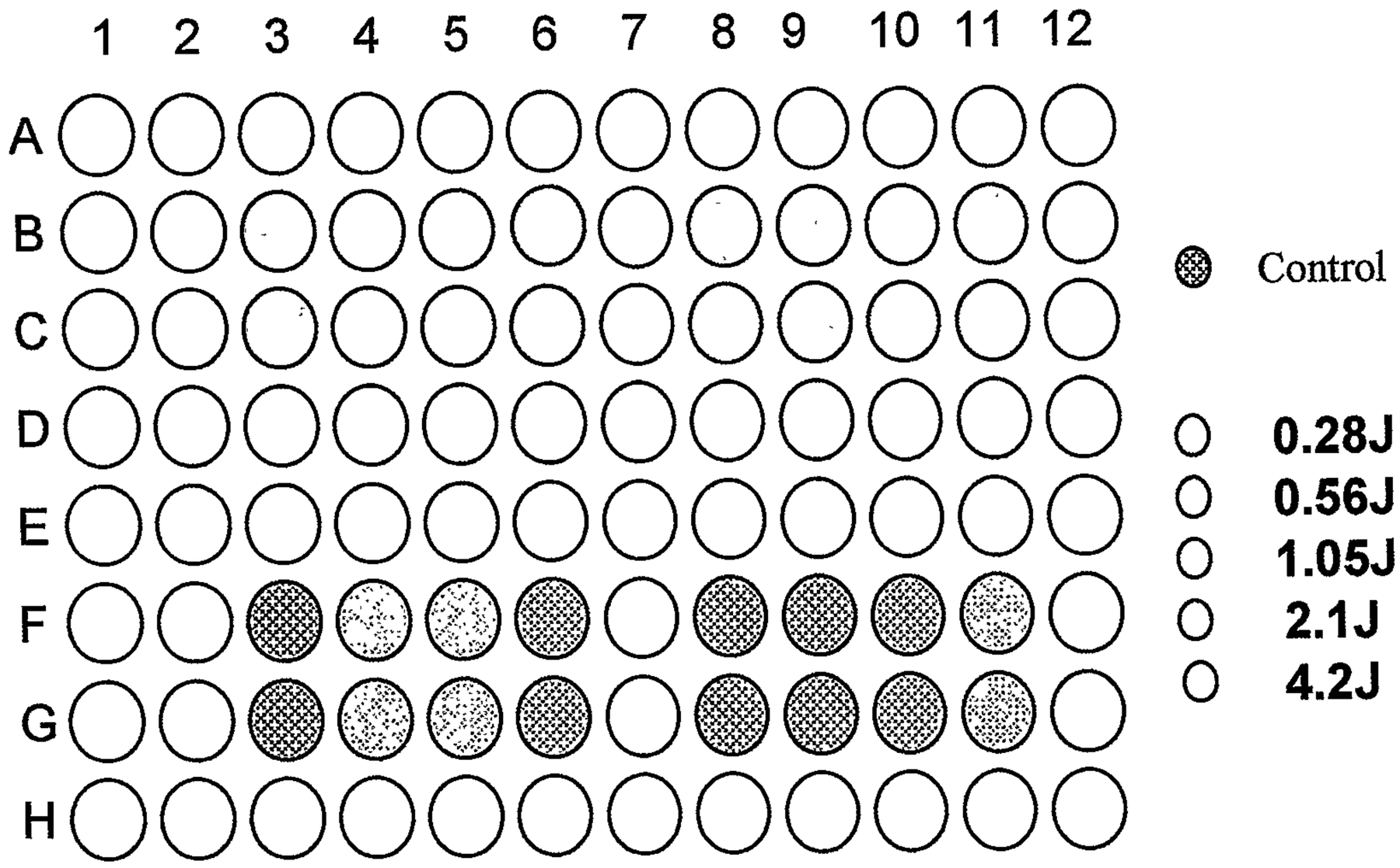

Figure 2.7. Well arrangement for pilot study (MTT assay)

2.9 Experiment 1: Proteins expression

Cells were seeded into 96 wells plates with a seeding density of 20,000 cells/well. The cells were irradiated 24 hours after seeding and then daily for another 3 days as shown in Figure 2.8. Cells were harvested for SDS-PAGE 24 hours after the second and fourth irradiations. Another group of plates was irradiated 48 hours after seeding and then daily for another 3 days as shown in Figure 2.8. Assay was done one hour after the second and fourth irradiations. Wells located in the same columns served as controls.

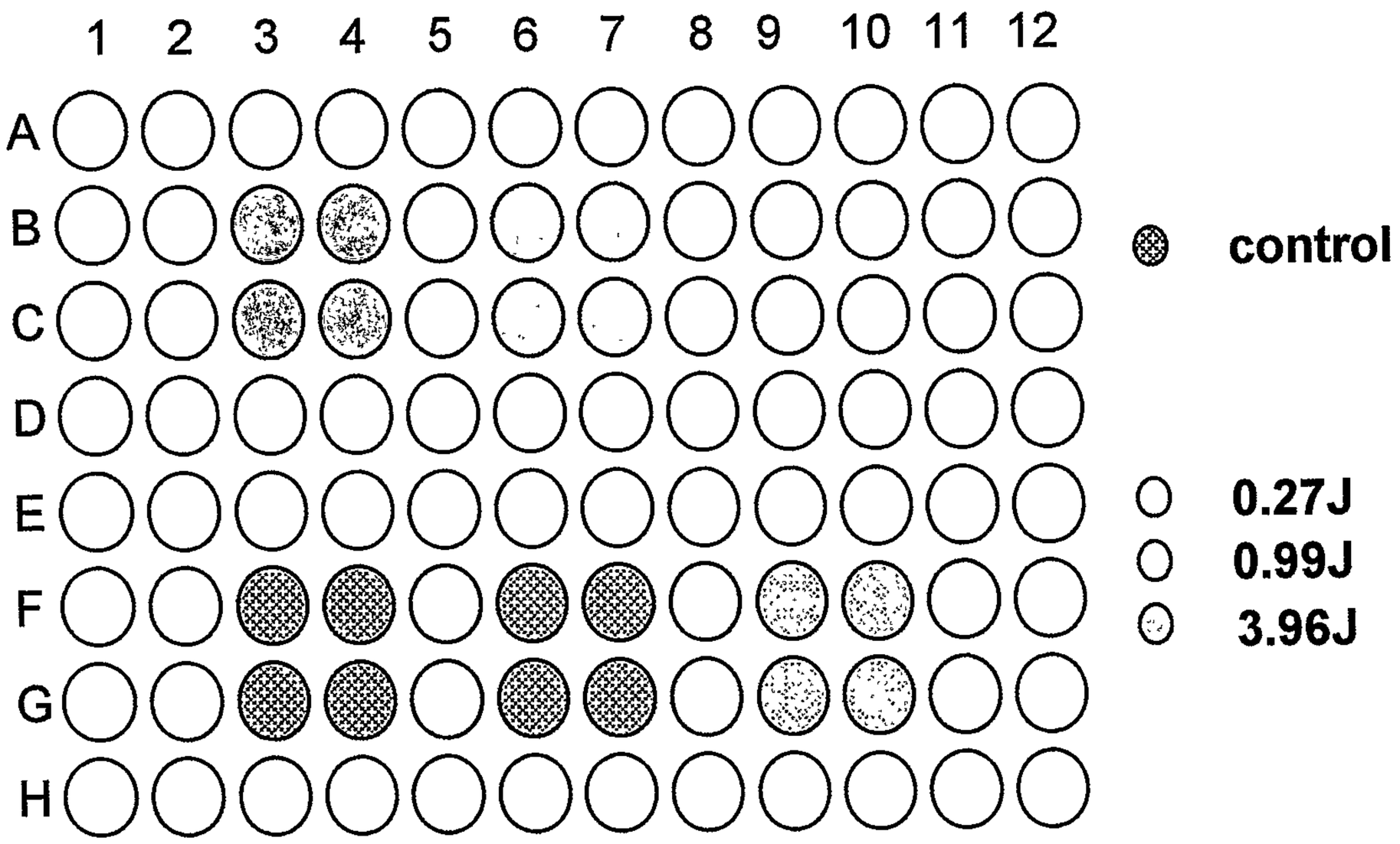

Figure 2.8. Well arrangement for SDS-PAGE assay

2.10 Experiment 2: Alkaline phosphatase activities

In order to determine the optimal seeding density and instrument parameters, ALP assay was performed on wells with different seeding density at different periods. Cells were seeded at 5,000, 10,000, 20,000, 40,000 cells/well as shown in Figure 2.9. ALP assay was performed on the fourth day after seeding according to protocol 1. Plates were also prepared at 5,000, 10,000, 15,000, 20,000 cells/well as shown in Figure 2.10. Medium was changed on day 3 and day 5. On the seventh day after seeding ALP assay was performed according to protocol 1.

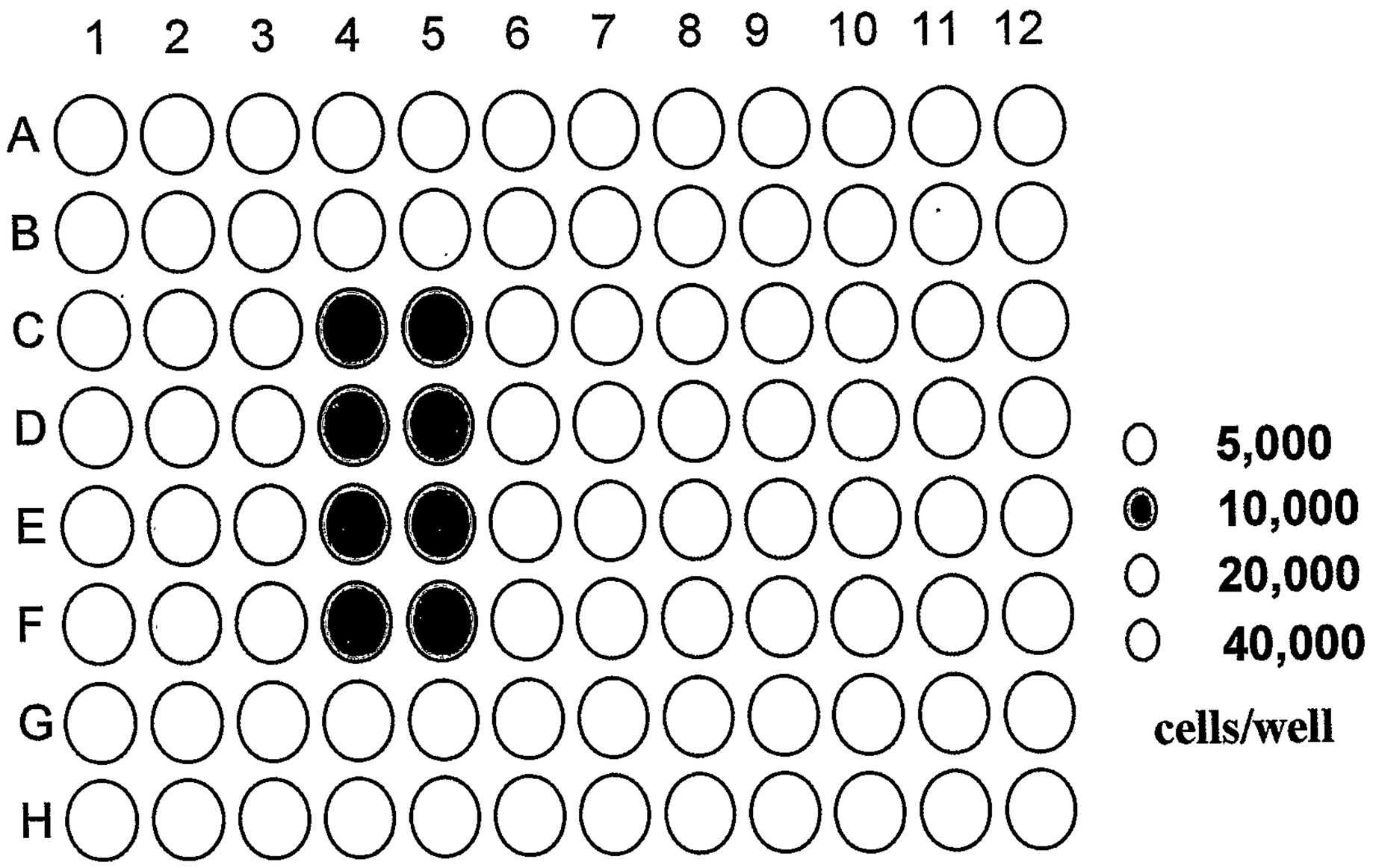

Figure 2.9. Well arrangement for ALP assay with 5,000-40,000 cells/well seeding densities

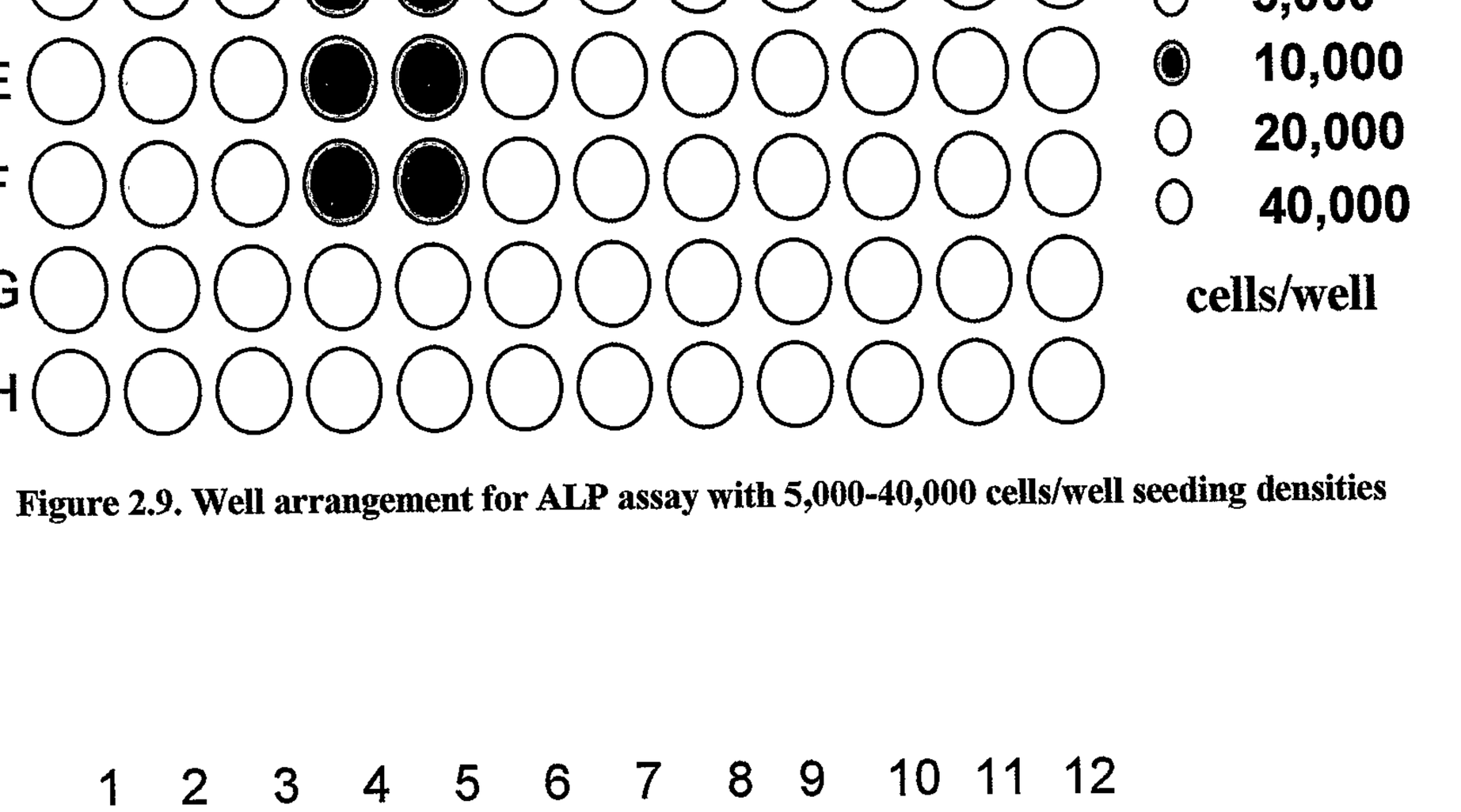

Figure 2.10. Well arrangement for ALP assay with 5,000-20,000 cells/well seeding densities

After the determination of optimal seeding density for ALP assay, cells were seeded at 10,000 cells/well. The cells were irradiated 24 hours after seeding and then daily for 7 days as shown in Figure 2.11. ALP assay was performed 24 hours after each irradiation according to protocol 1. Medium was changed on day 3 and day 5.

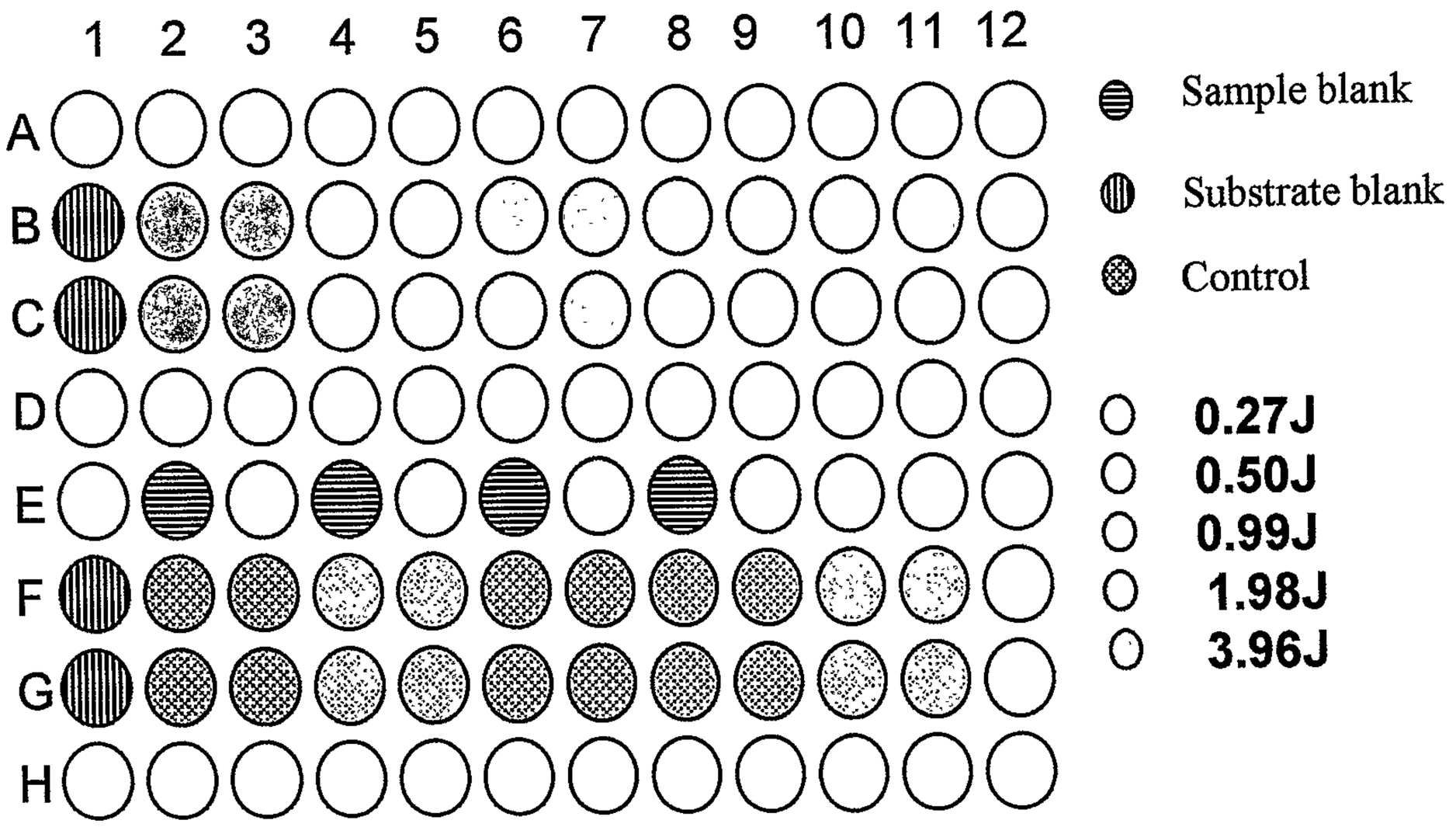

Figure 2.11. Well arrangement for ALP assay (5 energy levels)

Cells were later seeded at 2,500 cells/well. The cells were irradiated 48 hours after seeding and daily for 5 days as shown in Figure 2.11 and Figure 2.12. ALP assay was performed one hour after each irradiation according to protocol 2.

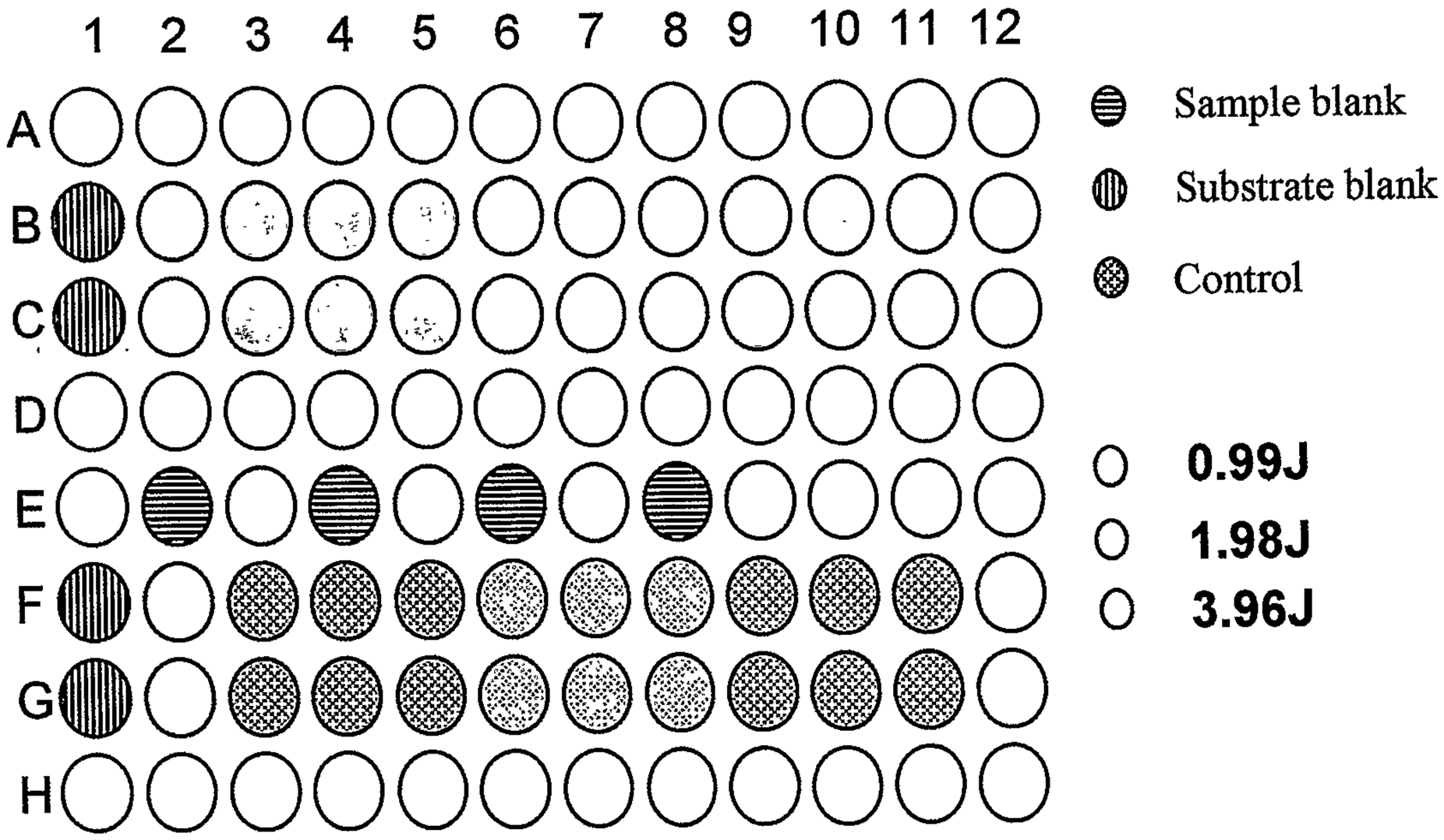

Figure 2.12. Well arrangement for ALP assay (3 energy levels)

2.11 Data consideration

2.11.1 Data collection

Data was collected from computer print outs and entered into MS Excel 7.

2.11.2 Data analysis

Data was analysed using SPSS 8 for Windows (SPSS). Descriptive statistics were used to describe mean and standard deviations. Linear regression was used to calculate rate of reaction of ALP activities. Independent t-tests were applied to irradiated and control groups in Experiment 2: ALP assay.

2.11.3 Data presentation

The graphs in Chapter Three were generated using SPSS viewer. The pictures of the mini-gels were produced through scanning with a flat top scanner and adjusting using Adobe Photoshop 2.5. Densitographs were hand-traced and superimposed. The superimposed figures were then scanned with a flat top scanner and adjusted using Adobe Photoshop 2.5.

CHAPTER THREE

RESULTS

3.1 Cell culture

SAOS-2 was a human osteosarcoma cell line. The cells grew as monolayer. Morphologically, they were epithelial-like. They could grow in low seeding densities. However, uneven spread of cells might be found with low seeding densities. Figure 3.1 shows the morphology of cells in low magnification under light microscope.

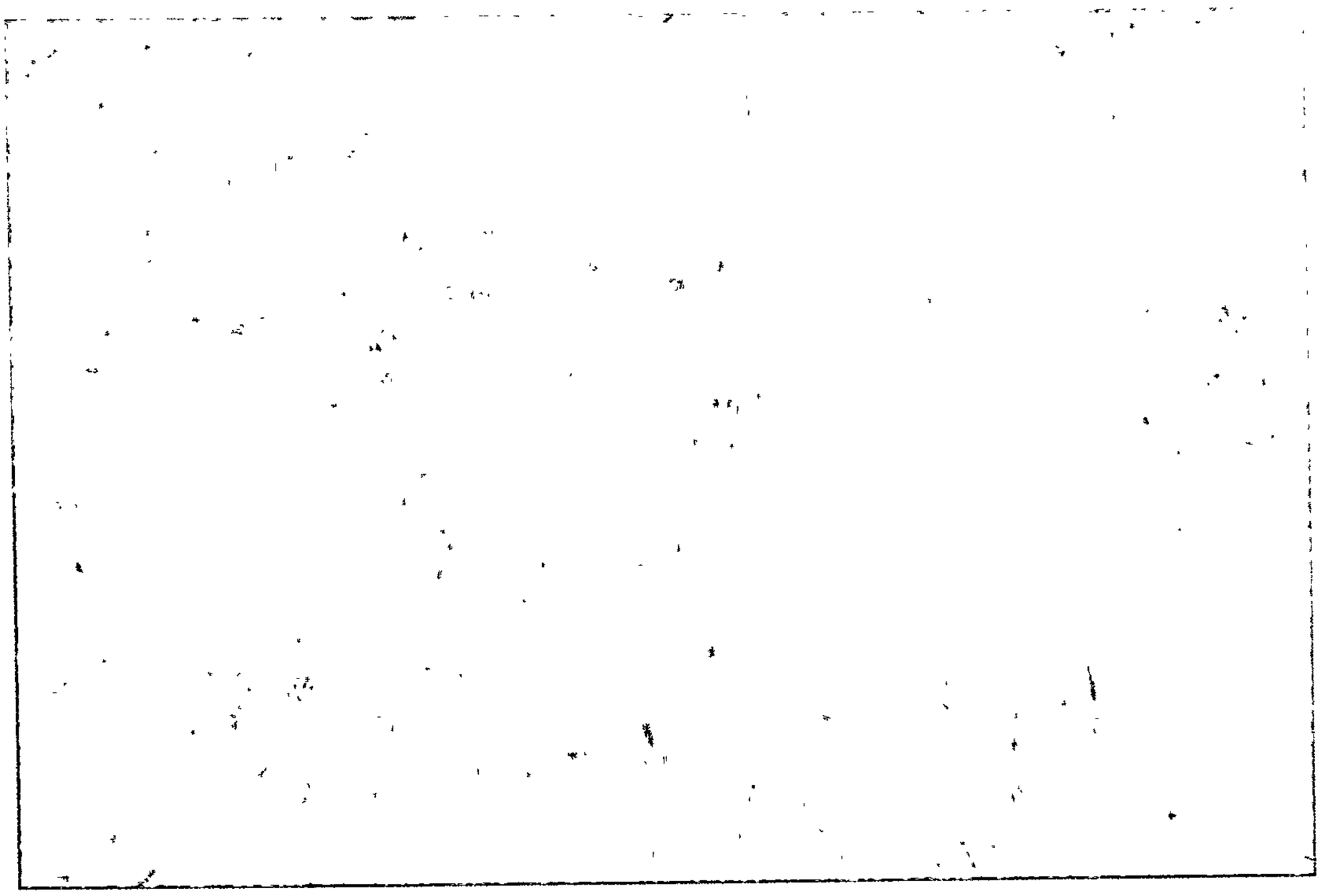

Figure 3.1. SAOS-2 under low magnification

3.2 Pilot Study

3.2.1 Seeding density

Table 3.1 summarised the MTT results with different seeding densities. The MTT results showed that seeding density with 10,000 cells/well was a suitable choice for the future experiments. The MTT results of 10,000 cells/well lay between those of 5,000 and 20,000 cells/well (Fig. 3.2). It allowed any positive and negative experimental changes to be recorded. The increase in MTT absorbance followed a normal growth curve.

Table 3.1. MTT results with different seeding densities

Day	5,000 cells/well	10,000 cells/well	15,000 cells/well	20,000 cells/well
1	0.113±0.018	0.183±0.019	0.231±0.001	0.191±0.043
2	0.098±0.029	0.173±0.055	0.195±0.062	0.234±0.038
3	0.140±0.013	0.201±0.015	0.224±0.012	0.256±0.022
4	0.211±0.012	0.286±0.017	0.343±0.028	0.341±0.034
5	0.261±0.011	0.362±0.023	0.414±0.018	0.385±0.029
6	0.256±0.009	0.346±0.014	0.436±0.014	0.497±0.003
7	0.274±0.037	0.369±0.031	0.417±0.026	0.515±0.022
8	0.391±0.099	0.498±0.155	0.594±0.136	0.696±0.075
9	0.581±0.029	0.549±0.190	0.78±0.092	0.636±0.22

3.2.2 Observation time

Table 3.2 summarised the MTT results of cells irradiated with different energy levels on six consecutive days. The standard deviation of the results was in the order of 5-10% (Tab. 3.2). Although it appeared that there was a drop of MTT absorbance on Day 4, it coincided with time of medium changes. There were no dose-related changes observed in the pilot study (Fig. 3.3). The time window for observable changes could not be determined.

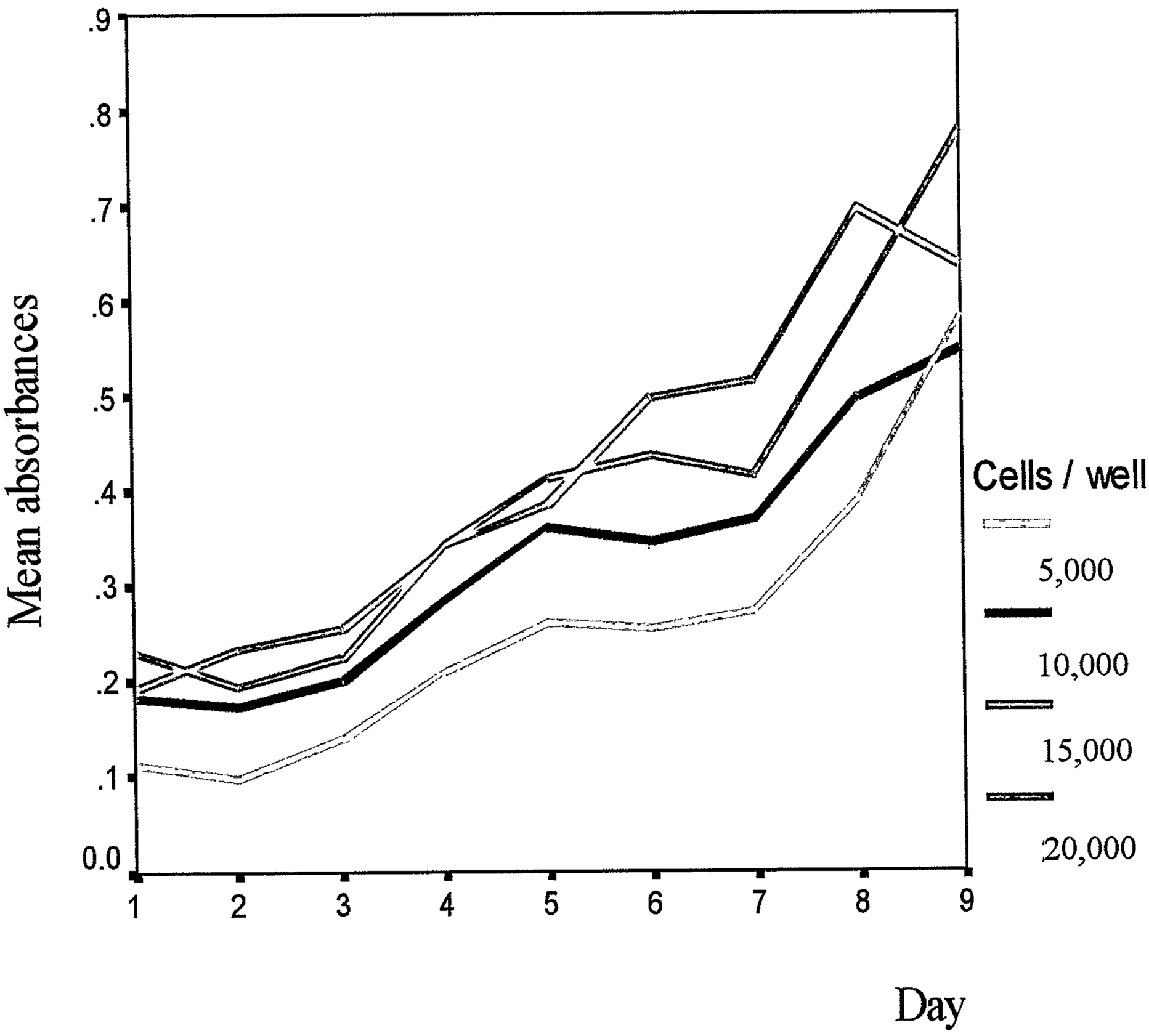

Figure 3.2. MTT results with different seeding densities

Table 3.2. MTT results with different energy levels

Day	0.28J	0.56J	1.05J	2.1J	4.2J
1	0.214±0.017	0.193±0.018	0.214±0.010	0.229±0.016	0.145±0.025
2	0.221±0.016	0.233±0.024	0.247±0.009	0.198±0.027	0.204±0.008
3	0.281±0.027	0.317±0.023	0.309±0.034	0.337±0.019	0.285±0.003
4	0.298±0.032	0.322±0.019	0.355±0.038	0.362±0.034	0.230±0.008
5	0.440±0.015	0.421±0.037	0.427±0.018	0.358±0.025	0.451±0.023
6	0.526±0.016	0.479±0.013	0.465±0.012	0.454±0.033	0.521±0.036

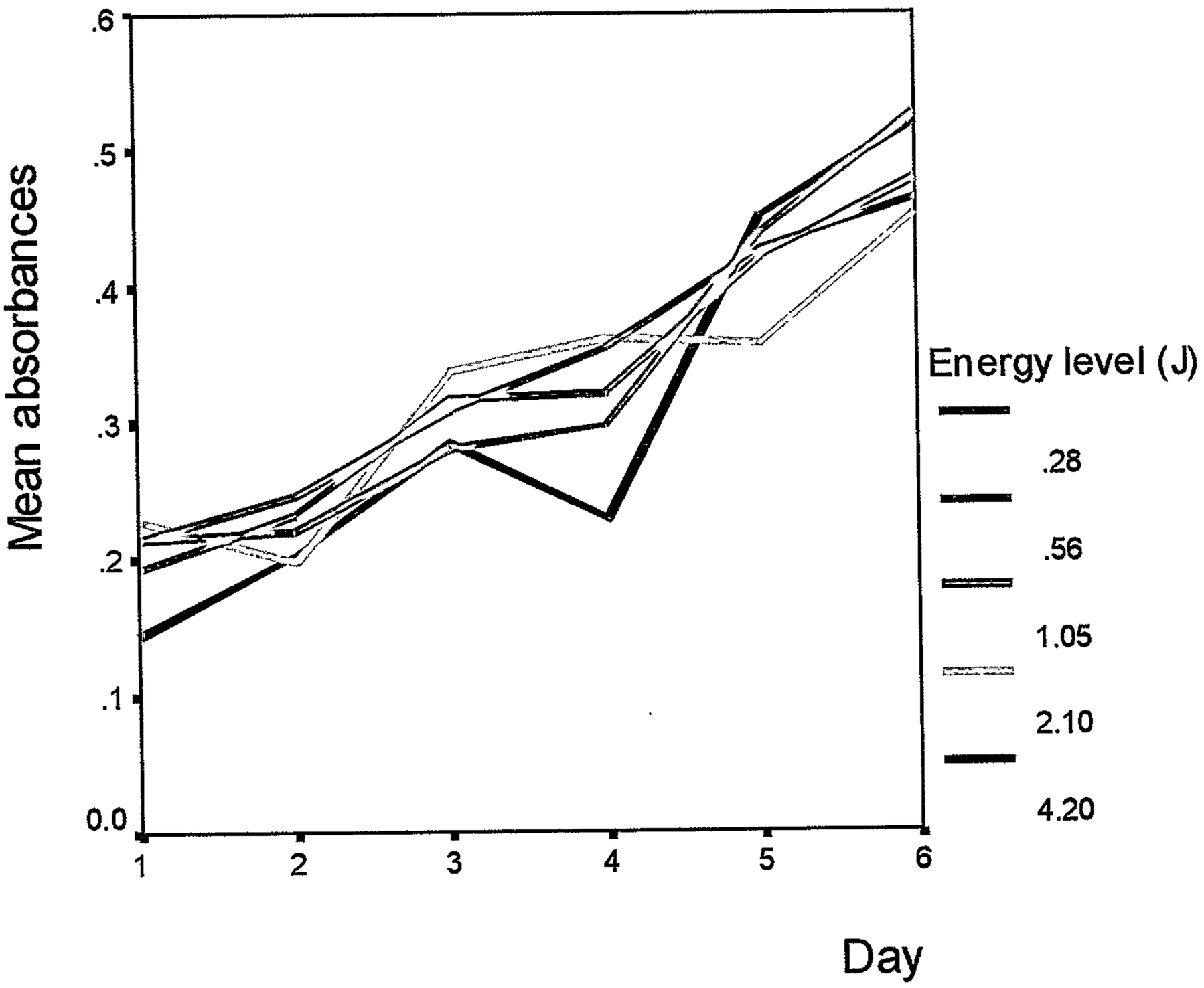

Figure 3.3. MTT results with different energy levels

3.3 Experiment 1: Protein expression

3.3.1 Late effects of laser irradiation

The first part of the experiment was to investigate whether 2 and 4 daily laser irradiation to SAOS-2 with energy levels 0.27J, 0.99J and 3.96J would produce

any late (24 hours later) changes in protein expression. Mini-gels of 2 daily irradiations were too faint to be scanned and are not shown here. Figure 3.4 shows the mini-gel stained with Coomassie blue, which was loaded with cell extract 24 hours after 4 daily laser irradiations. Figures 3.5 shows the same gel stained with silver stain. Table 3.3 lists the keys of the column of Figures 3.4 and 3.5. Figures 3.6 and 3.7 are pictures of replicates. Table 3.4 lists the keys of columns of Figures 3.6 and 3.7. Table 3.5 lists the molecular weight of the standard. Although the background of the silver stained gels was overstained, the patterns of band were clearly seen. More bands were found in silver stained gels compared with gels stained with Coomassie blue, especially at the top ends. Thick bands were not found around the 2nd band of the standard (bovine serum albumin, MW: 81,000). This showed that contamination due to foetal calf serum from the medium was minimal. The assay showed no observable differences in band pattern between the irradiated and control groups on visual inspection (with assistance from Derek W.S. Harty, Senior Scientific Officer, Institute of Dental Research, United Dental Hospital).

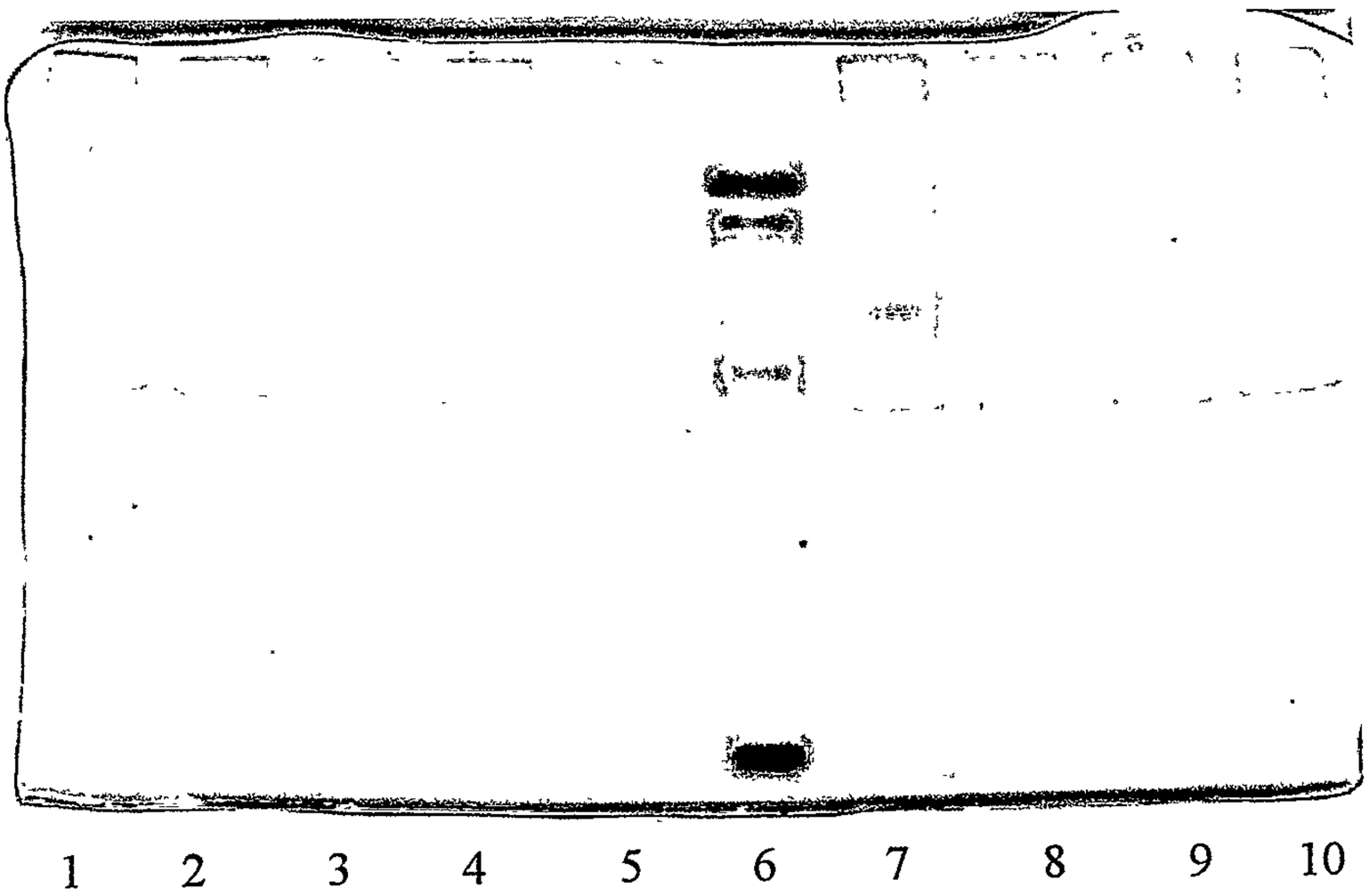

Figure 3.4. Coomassie stained mini-gel: 24 hours after irradiation

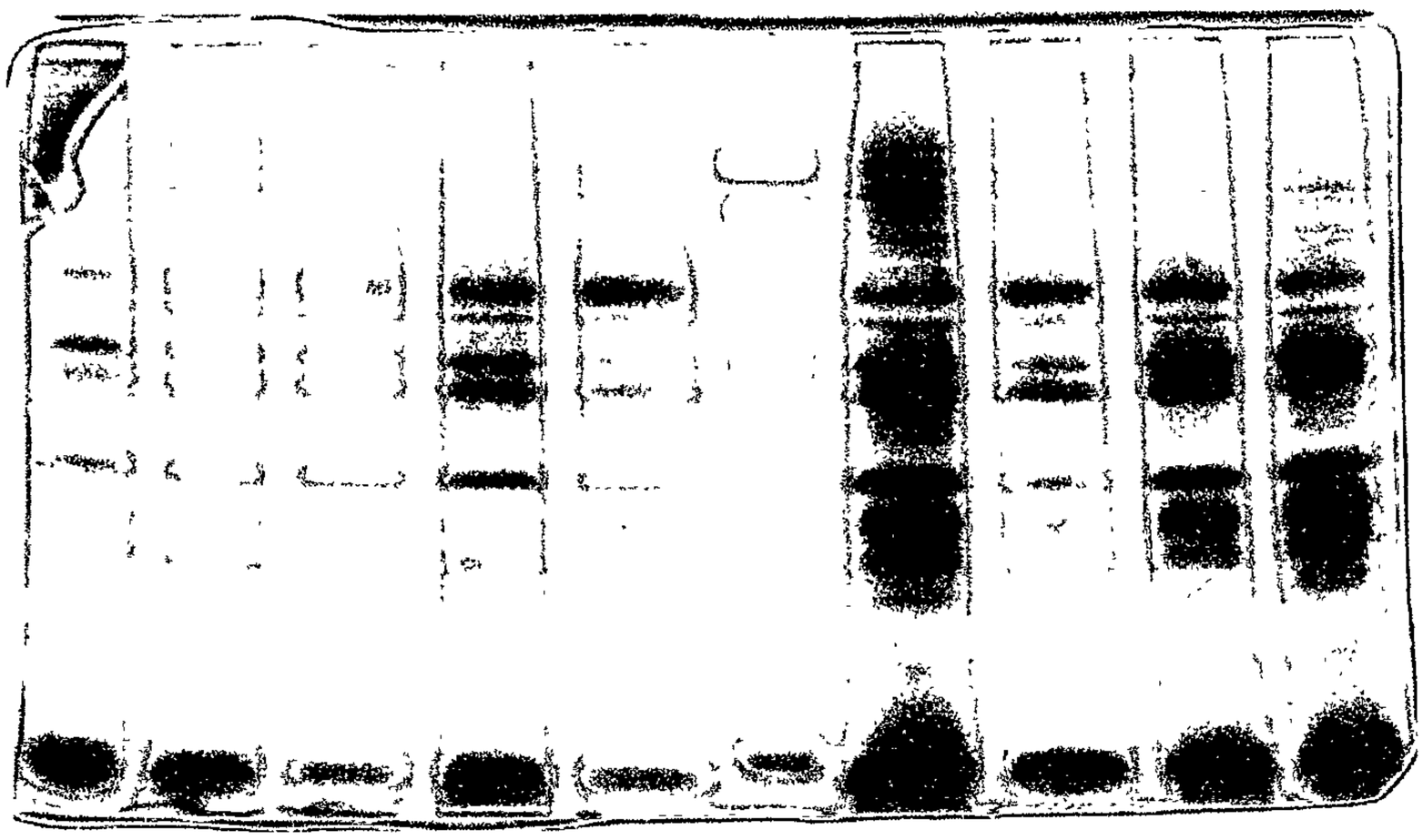

Figure 3.5. Silver stained mini-gel: 24 hours after irradiation

Table 3.3. Keys for Figures 3.4 and 3.5

Column	Keys	Column	Keys
1	0.27J irradiation	6	Standard
2	0.27J irradiation	7	0.99J irradiation
3	0.27J control	8	3.96J irradiation
4	0.99J irradiation	9	3.96J control
5	0.99J control	10	3.96J irradiation

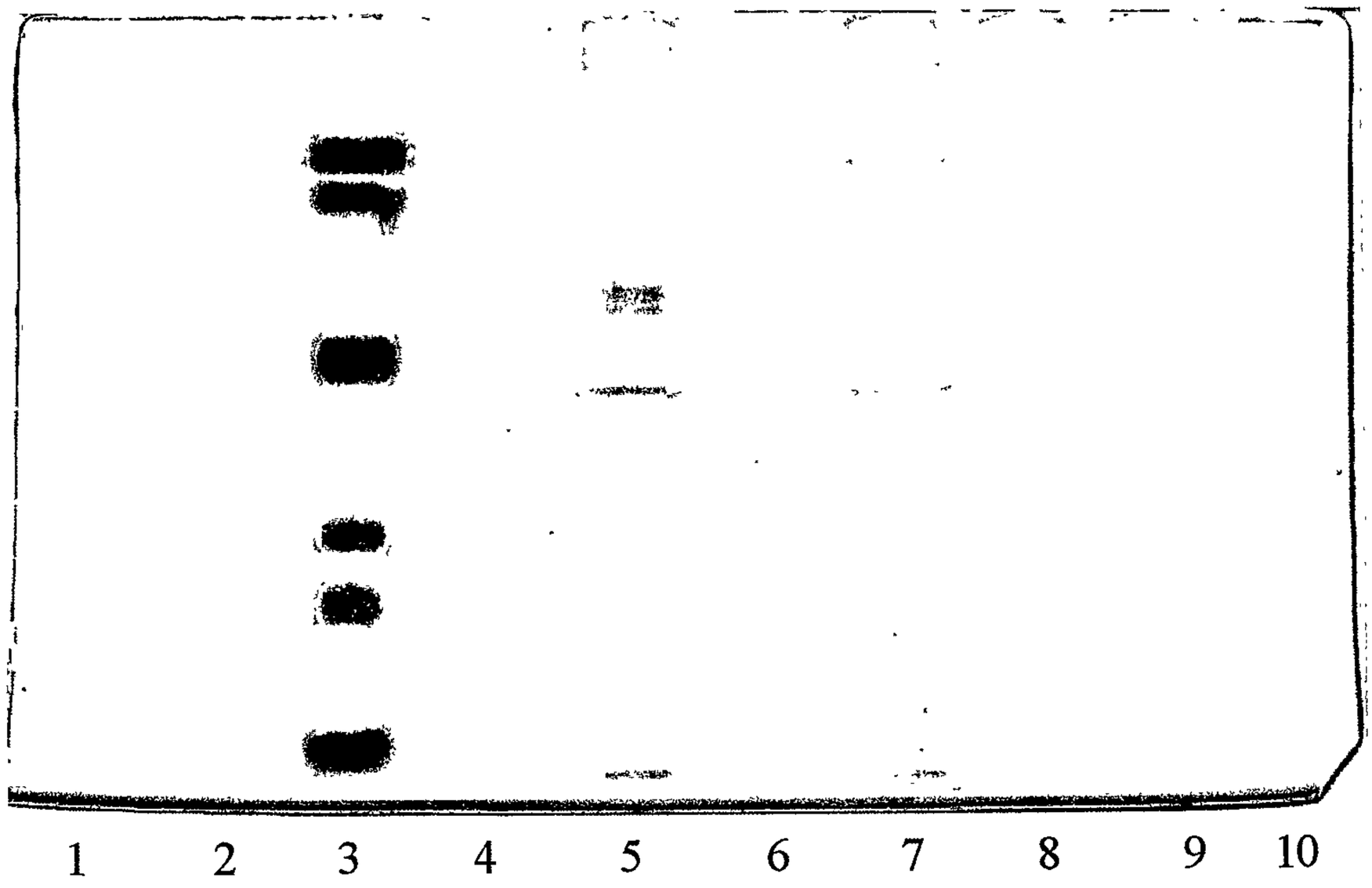

Figure 3.6. Coomassie stained mini-gel (replicate): 24 hours after irradiation

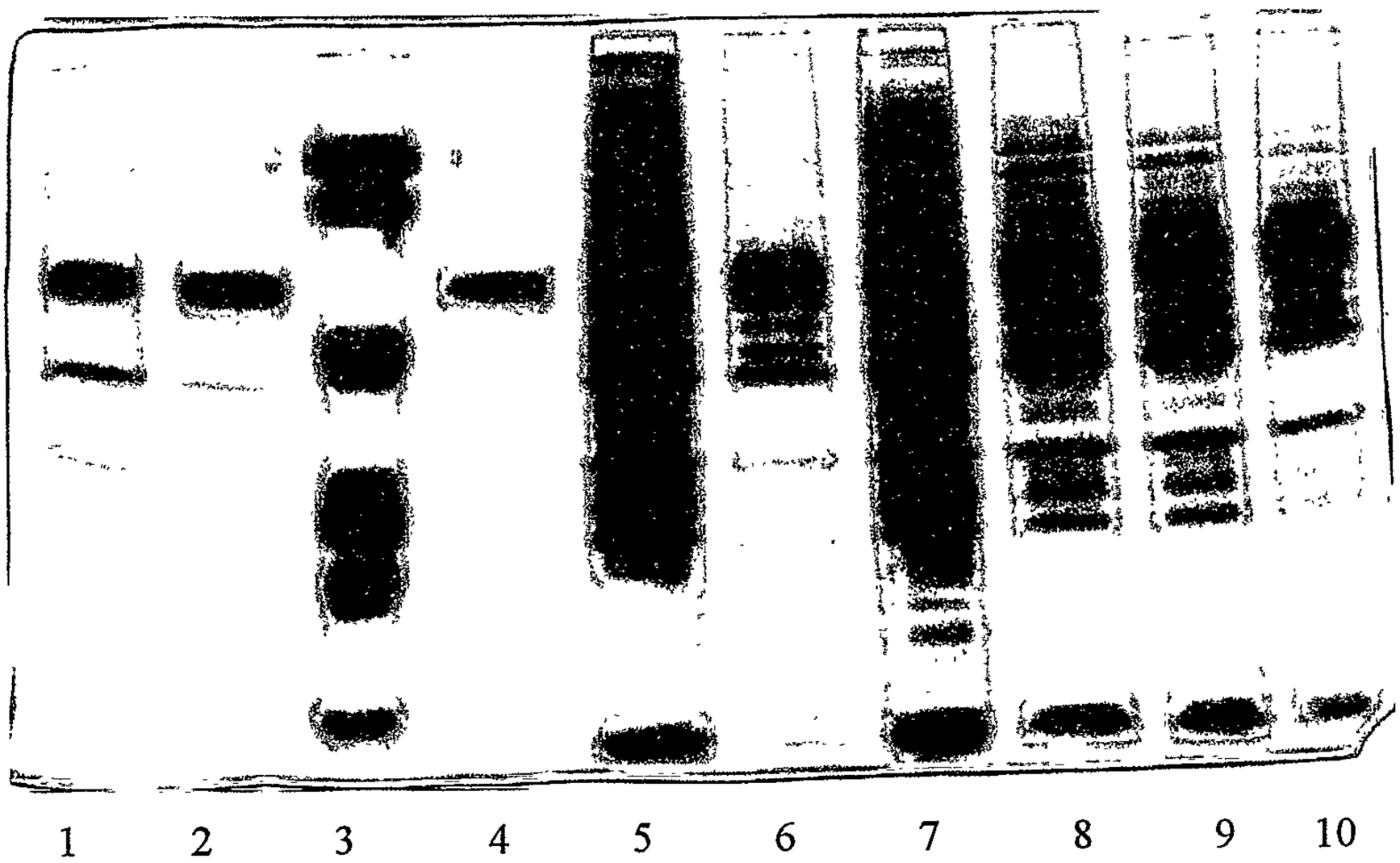

Figure 3.7. Silver stained mini-gel (replicate): 24 hours after irradiation

Table 3.4. Keys for Figures 3.6 and 3.7

Column	Keys	Column	Keys
1	0.27J control	6	0.99J control
2	Loading error	7	0.99J control
3	Standard	8	3.96J irradiation
4	Loading error	9	3.96J control
5	0.99J irradiation	10	3.96J irradiation

Table 3.5. Standard for mini-gels (low range, Bio-Rad Laboratories, USA)

Standard	MW (daltons)
Phosphorylase B	102,000
Bovine serum albumin	81,000
Ovalbumin	46,900
Carbonic anhydrase	32,700
Soybean trypsin inhibitor	30,200
Lysozyme	24,000

The mini-gels stained with Coomassie blue were scanned with a PC flat top scanner. A black and white printout was used for densitometry scanning. The irradiated and corresponding control columns were scanned by densitometer with the same setting. The vertical distance of the bands from the top of the scanning panel of the densitometer was called the Y distance. In order to minimise the background noise and to smooth the line of the graphs, the mini-gels were scanned every 4-unit distance (manufacturer setting) along the Y distance i.e. Y-step was set at 4. The Y distance of the bands was plotted at X axis in the densitographs. The results of the irradiated and control groups were superimposed for easy comparison. Figure 3.8 shows the densitograph of the mini-gel with cell extract 24 hours after 4 daily 3.69J laser irradiations.

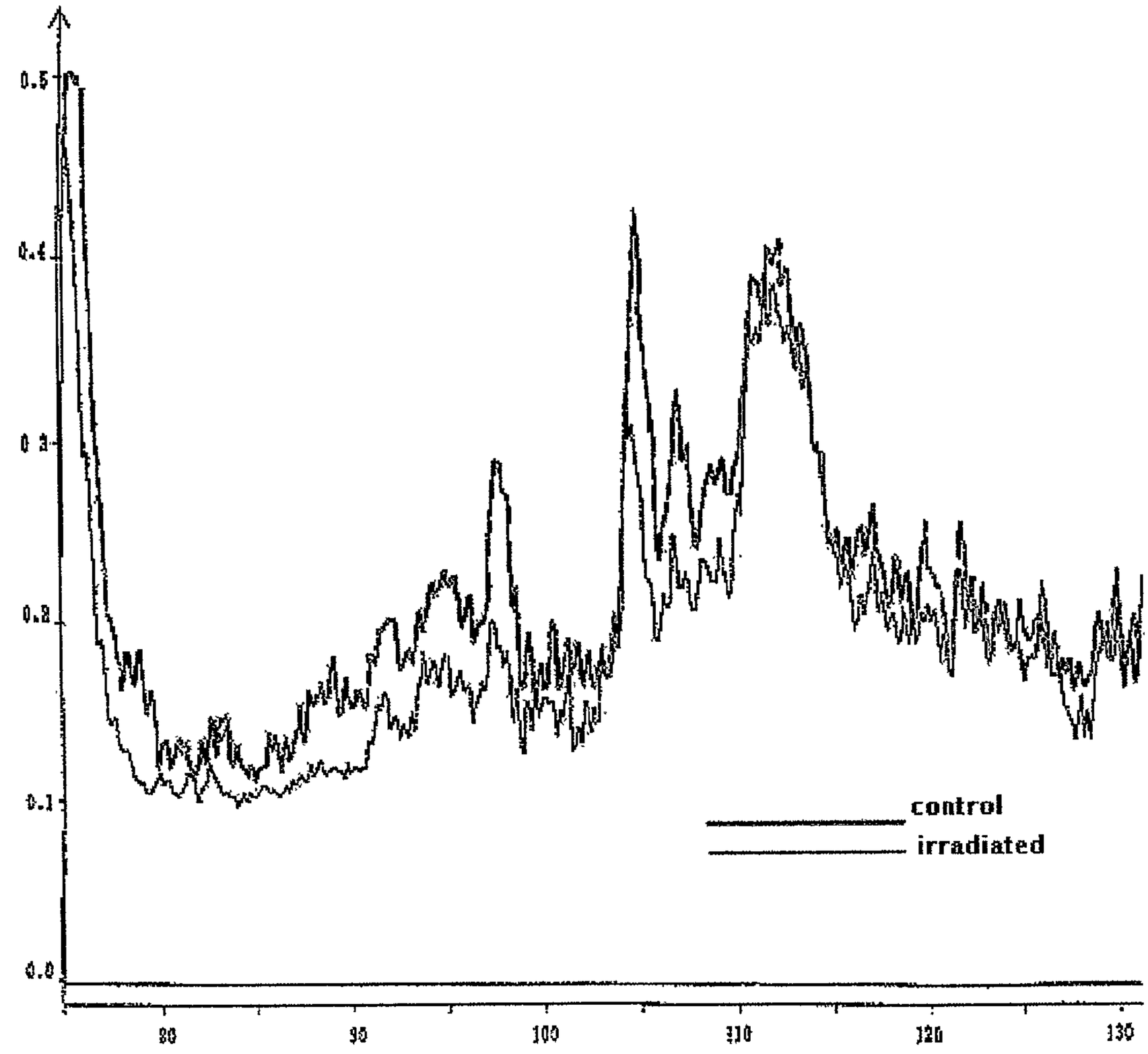

Figure 3.8. Densitograph of the irradiated sample with 4 daily 3.96J laser irradiations compared with the control sample (24 hours after)

The results of Coomassie stained were quantitative (Laemmli 1970). The results of densitometry were expected to be quantitative. The distribution and magnitude of peaks in the densitograph was very similar between the irradiated and control groups. The differences in density observed in some of the peaks were probably related to differences in cell number during harvesting and/or in loading volume during the assay. The results including those from replicates showed that there was no significant difference between the irradiated and control groups in different energy levels. Therefore, it was concluded that 4 daily laser irradiations with energy levels 0.27J, 0.99Jand 3.96J did not produce any significant long term changes (24 hours later) in protein expression.

3.3.2 Early effects of laser irradiation

The second part of the assay was to investigate whether 2 and 4 daily irradiations to SAOS-2 would produce any early (1 hour) changes. Mini-gels of 2 daily irradiations were too faint to be scanned and were not shown here. Figure 3.9 shows the mini-gel stained with Coomassie blue, which were loaded with cell extract 1 hour after 4 daily laser irradiations. Figures 3.10 shows the same gel stained with silver stain. Table 3.6 lists the keys of the columns of Figures 3.9 and 3.10. Figures 3.11 and 3.12 show the replicates. Table 3.7 lists the keys of column of Figures 3.11 and 3.12.

Figure 3.9. Coomassie stained mini-gel: 1 hour after irradiation

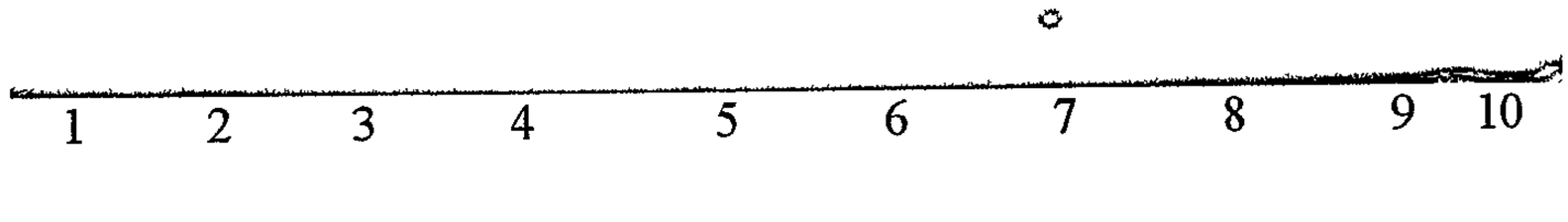

Figure 3.10. Silver stained mini-gel: 1 hour after irradiation

Table 3.6. Keys for Figures 3.9 and 3.10

Column	Keys	Column	Keys
1	Blank	6	0.99J irradiation
2	3.96J irradiation	7	0.99J control
3	3.96J control	8	0.27J irradiation
4	Blank	9	0.27J control
5	Standard	10	Blank

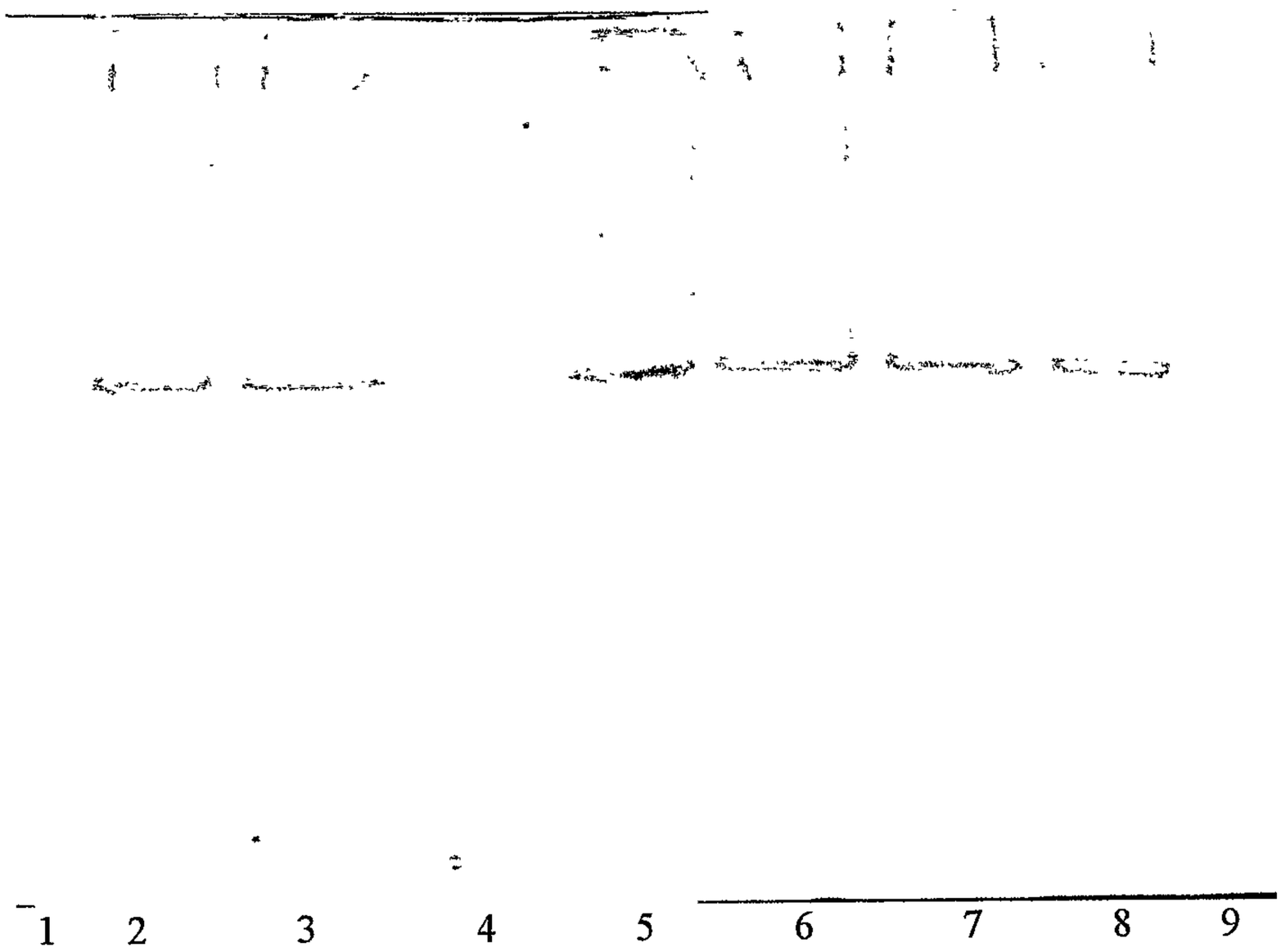

Figure 3.11. Coomassie stained mini-gel (replicate): 1 hour after irradiation

1 2 3 4 5 6 7 8 9

Figure 3.12. Silver stained mini-gel (replicate): 1 hour after irradiation

Table 3.7. Keys for Figures 3.11 and 3.12

Column	Keys	Column	Keys
1	Blank	6	0.99J control
2	0.27J irradiation	7	3.96J irradiation
3	0.27J control	8	3.96J control
4	Standard	9	Blank
5	0.99J irradiation		

Similar bands had been observed in the Coomassie and silver stained gels. Although the silver stained gels might be slightly understained, the background was clear and the patterns of band were clearly seen. Thick bands were not found around the 2nd band of the standard (bovine serum albumin, MW: 81,000). It showed that contamination due to foetal calf serum from the medium was minimal. The assay showed no observable differences in band pattern between the irradiated and control groups on visual inspection. Figure 3.13 to 3.15 show the densitographs of the mini-gels with cell extract 1 hour after 4 daily 0.27-3.69J laser irradiations. The results of the irradiated and control groups were superimposed for easy comparison.

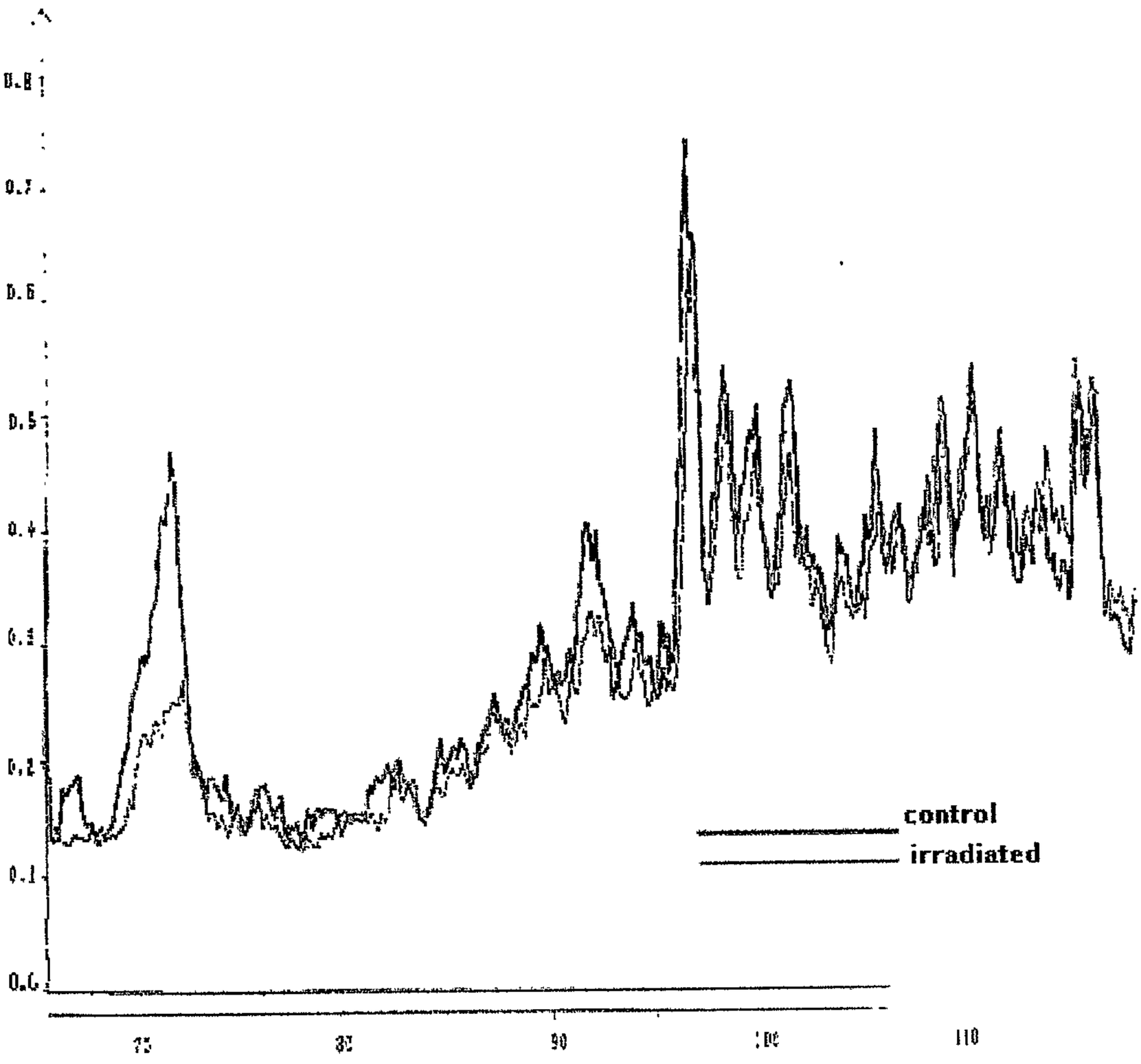

Figure 3.13. Densitograph of the irradiated sample with 4 daily 0.27J laser irradiations compared with the control sample (1 hour after)

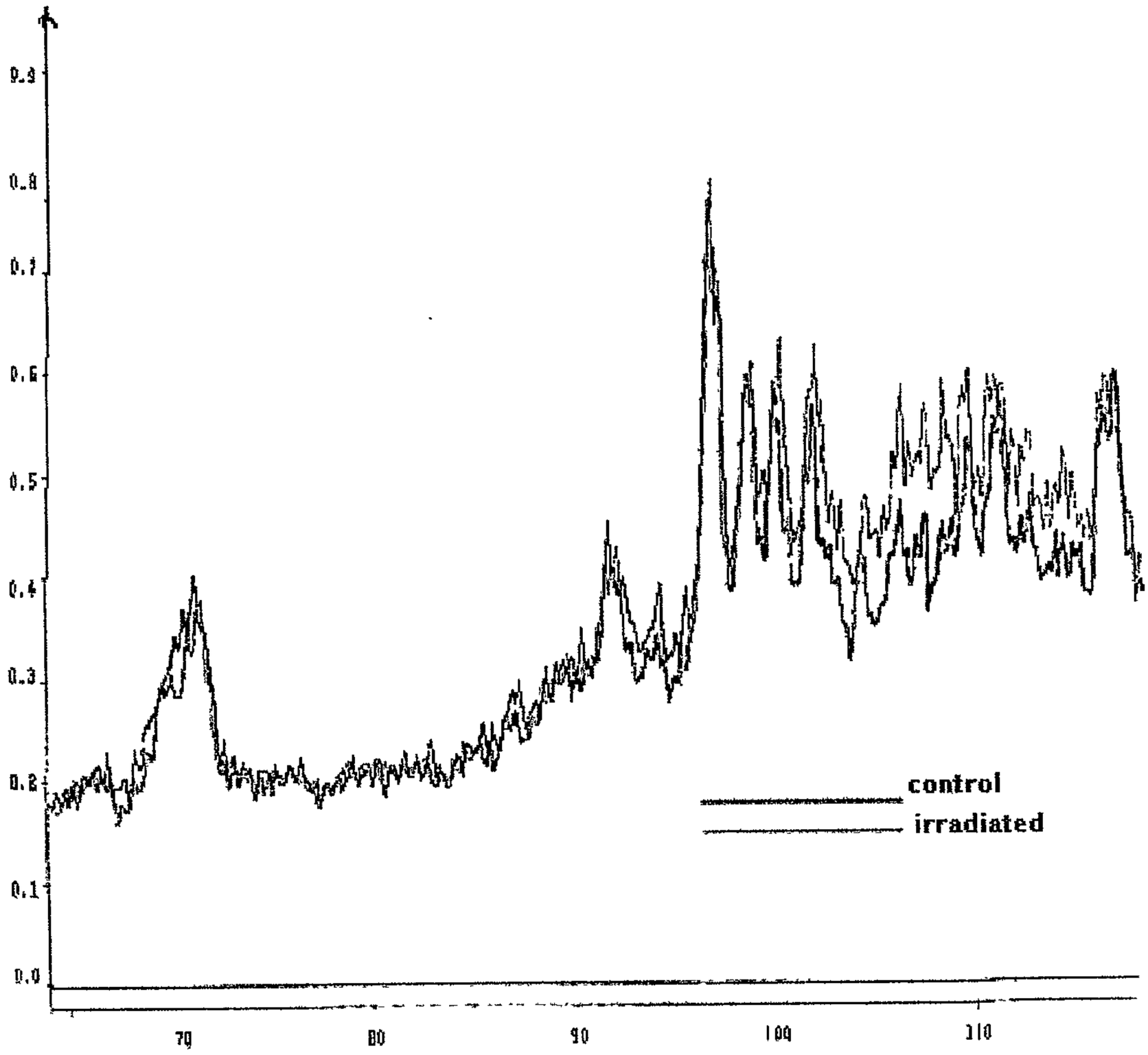

Figure 3.14. Densitograph of the irradiated sample with 4 daily 0.99J laser irradiations compared with the control sample (1 hour after)

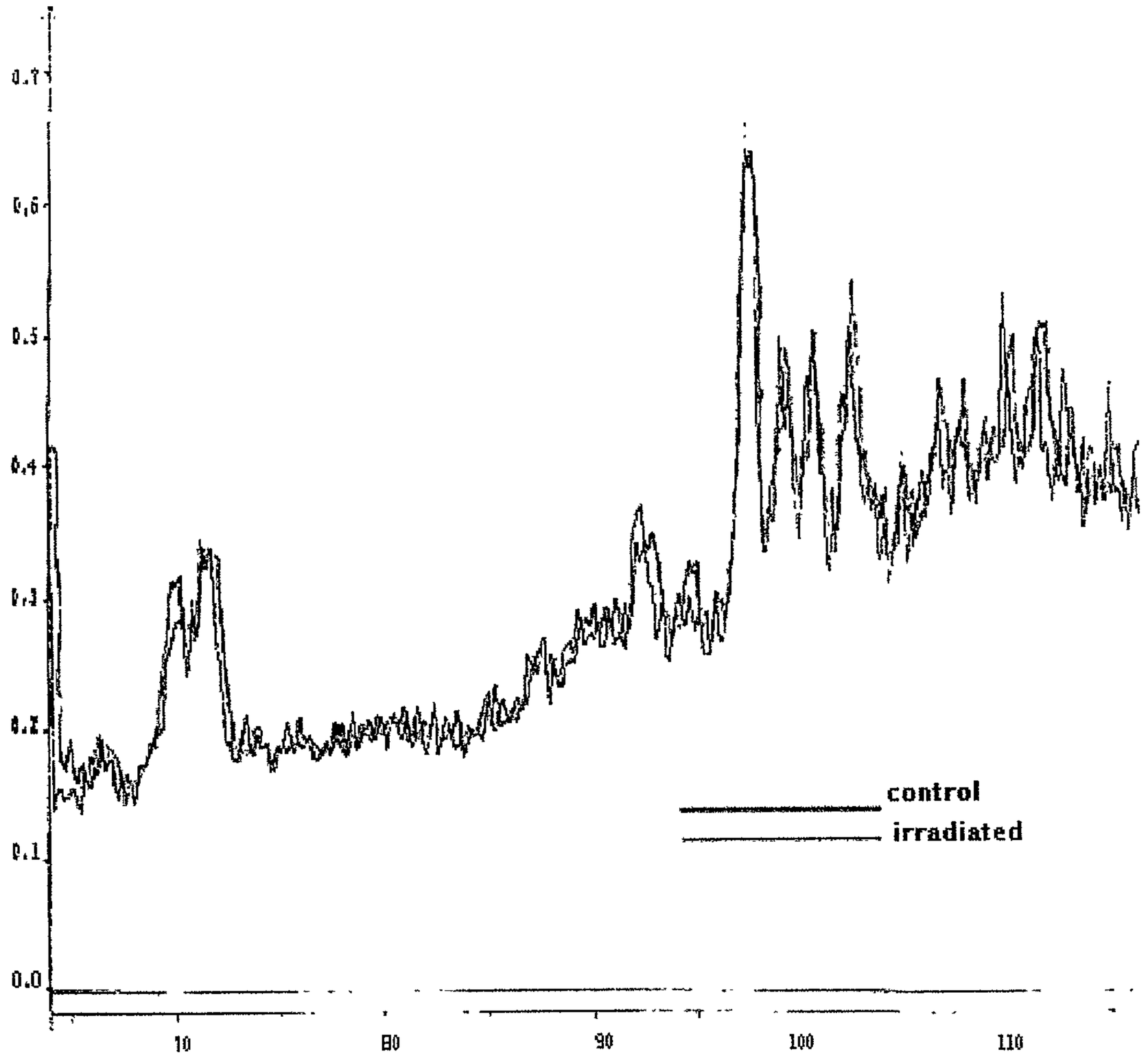

Figure 3.15. Densitograph of the irradiated sample with 4 daily 3.96J laser irradiations compared with the control sample (1 hour after)

The results of the densitometry showed that the graphs of the irradiated group and the corresponding controls had the same patterns and similar density. The two patterns matched very well. No significant differences between the irradiated group and the controls were found. Therefore, it is concluded that 4 daily laser irradiations with energy levels 0.27J, 0.99J and 3.96J did not produce any significant early changes (1 hour later) in protein expression. Daily laser irradiation with 0.27J, 0.99J and 3.96J for 4 days failed to produce significant early or late effects in protein expression in the present experimental set-up.

3.4 Experiment 2: ALP assay

3.4.1 Standards

Ten mM p-nitrophenol (Sigma, USA) was prepared as stock solution. It was diluted to 0.5mM and 2.5mM stock preparation. Different concentrations of p-nitrophenol was prepared (with the total volume as 100μL) into a 96 wells plate as in Table 3.8 and the plate was read by spectrophotometer. (Titertek Twinreader® Plus, EFLAB, Finland). The standard was used in protocol 1. Another standard was prepared (with total volume as 150ul) as in Table 3.9. The latter standard was used in protocol 2.

Table 3.8. p-nitrophenol standard preparation (total volume: 100μL)

p-nitrophenol (mM)	0.5mM	2.5mM	PBS	Buffer (pH 10.5)
0.000	0μL	----	46μL	54μL
0.025	5μL	----	41μL	54μL
0.050	10μL	----	36μL	54μL
0.075	15μL	----	31μL	54μL
0.100	20μL	----	26μL	54μL
0.150	30μL	----	16μL	54μL
0.200	----	8μLl	38μL	54μL
0.250	----	10μL	36μL	54μL
0.300	----	12μL	34μL	54μL
0.350	----	14μL	32μL	54μL
0.400	----	16μL	30μL	54μL

Table 3.9. p-nitrophenol standard preparation (total volume: 150μL)

p-nitrophenol (mM)	0.5mM	2.5mM	PBS	Buffer (pH 10.5)	1M NaOH
0.000	0μL	----	46μL	54μL	50μL
0.025	7.5μL	----	38.5μL	54μL	50μL
0.050	15μL	----	31μL	54μL	50μL
0.075	22.5μL	----	23.5μL	54μL	50μL
0.100	30μL	----	16μL	54μL	50μL
0.150	----	9μL	37μL	54μL	50μL
0.200	----	12μL	34μL	54μL	50μL
0.250	----	15μL	31μL	54μL	50μL
0.300	----	18μL	28μL	54μL	50μL
0.350	----	21μL	25μL	54μL	50μL
0.400	----	24μL	22μL	54μL	50μL

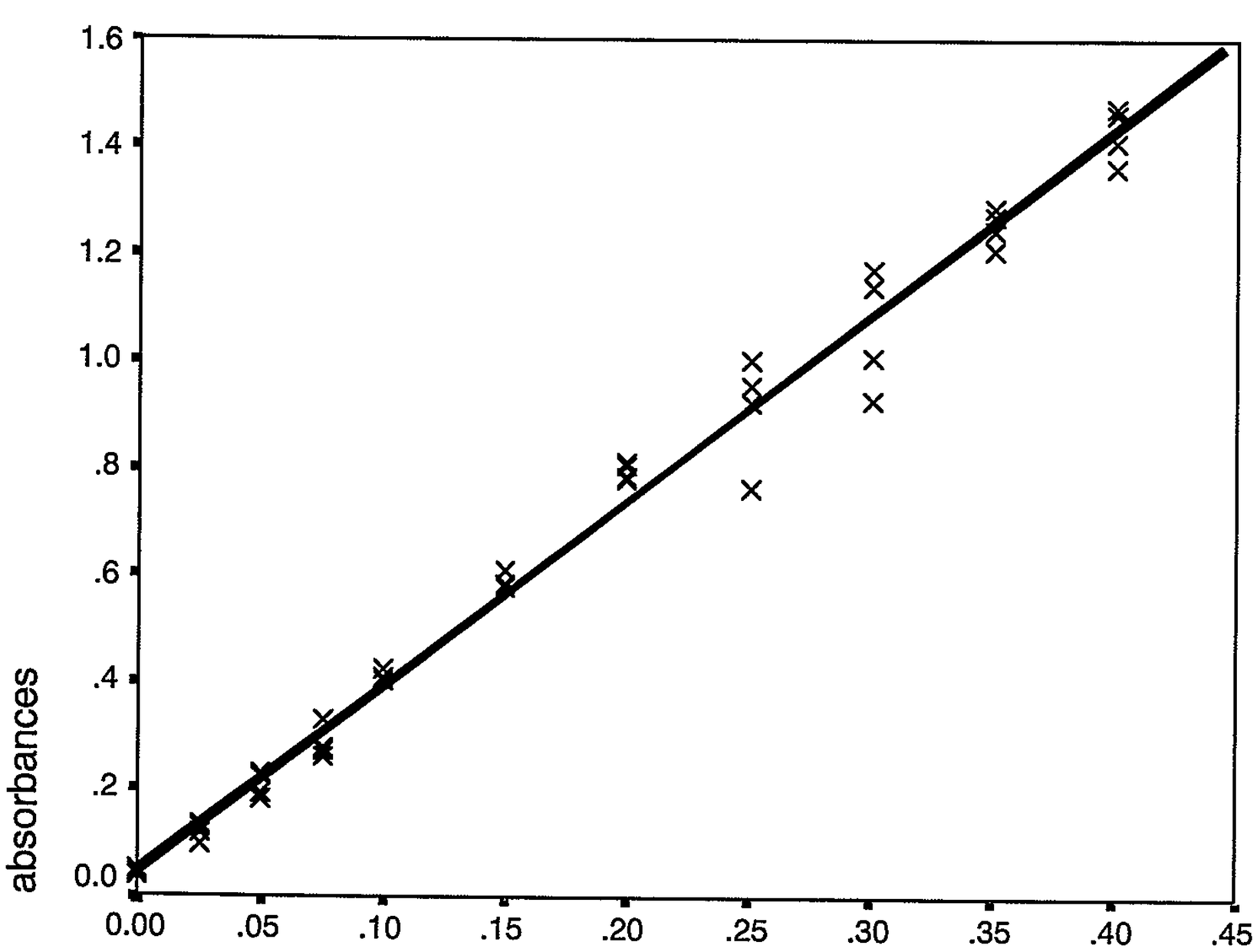

Figure 3.16. p-nitrophenol standard (total volume: 100μL)

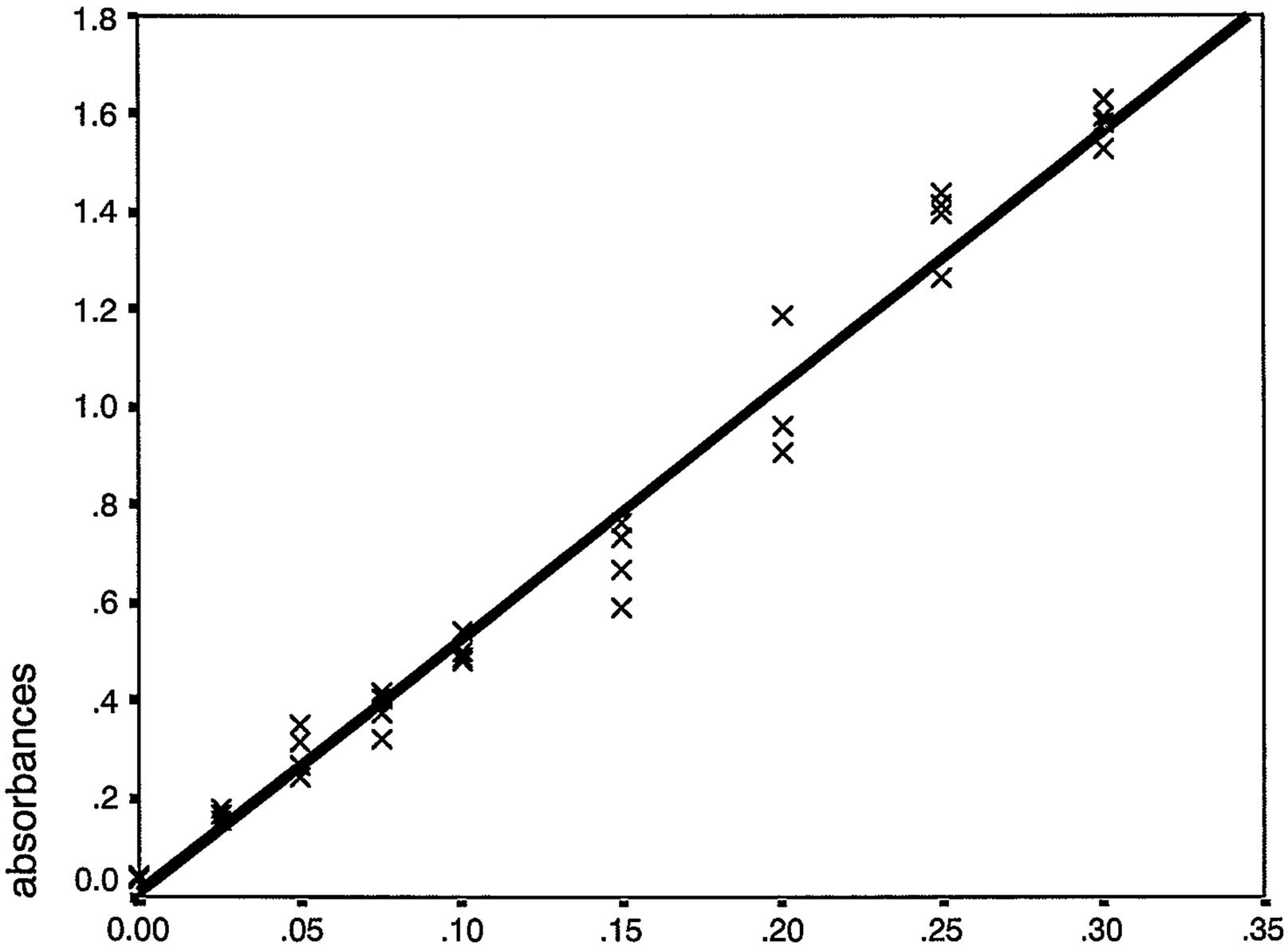

Figure 3.17. p-nitrophenol standard (total volume: 150μL)

The results of the p-nitrophenol standard are shown in Figures 3.16 and 3.17. It was found that the concentration of the p-nitrophenol standard was directly proportional to the absorbance at 405nm. The formula for Figure 3.6 was Y=3.460X+0.045. The formula for Figure 3.17 was Y=5.174X+0.009. The interceptions of both graphs were close to zero. Alkaline phosphatase activities were expressed as p-nitrophenol liberated per minutes. Since the absorbance at 405nm was directly proportional to the concentration of the standard (Fig. 3.16 and 3.17), the ALP activities of the following experiments were expressed as the increase in absorbance per minutes.

3.4.2 Seeding density

ALP activities were investigated on controls with different seeding densities. The cells were analysed after 4 days of incubations. The absorbance was measured every minute for 10 minutes. The results were shown in Table 3.10 and Figure 3.18.

Table 3.10. ALP assay results after 4 days of incubation

Time	5,000 cells/well	10,000 cells/well	20,000 cells/well	40,000 cells/well
1	0.127±0.091	0.457±0.247	0.554±0.230	0.824±0.096
2	0.132±0.115	1.249±0.363	1.468±0.374	2.091±0.046
3	0.310±0.259	1.879±0.367	2.176±0.339	2.501±0.116
4	0.613±0.315	2.356±0.172	2.545±0.161	2.629±0.108
5	0.863±0.313	2.566±0.067	2.640±0.133	2.658±0.096
6	1.156±0.335	2.636±0.070	2.670±0.123	2.716±0.083
7	1.442±0.357	2.640±0.076	2.670±0.118	2.713±0.084
8	1.728±0.365	2.639±0.095	2.684±0.135	2.716±0.076
9	1.944±0.354	2.651±0.092	2.690±0.132	2.739±0.057
10	2.160±0.314	2.663±0.093	2.698±0.134	2.730±0.085

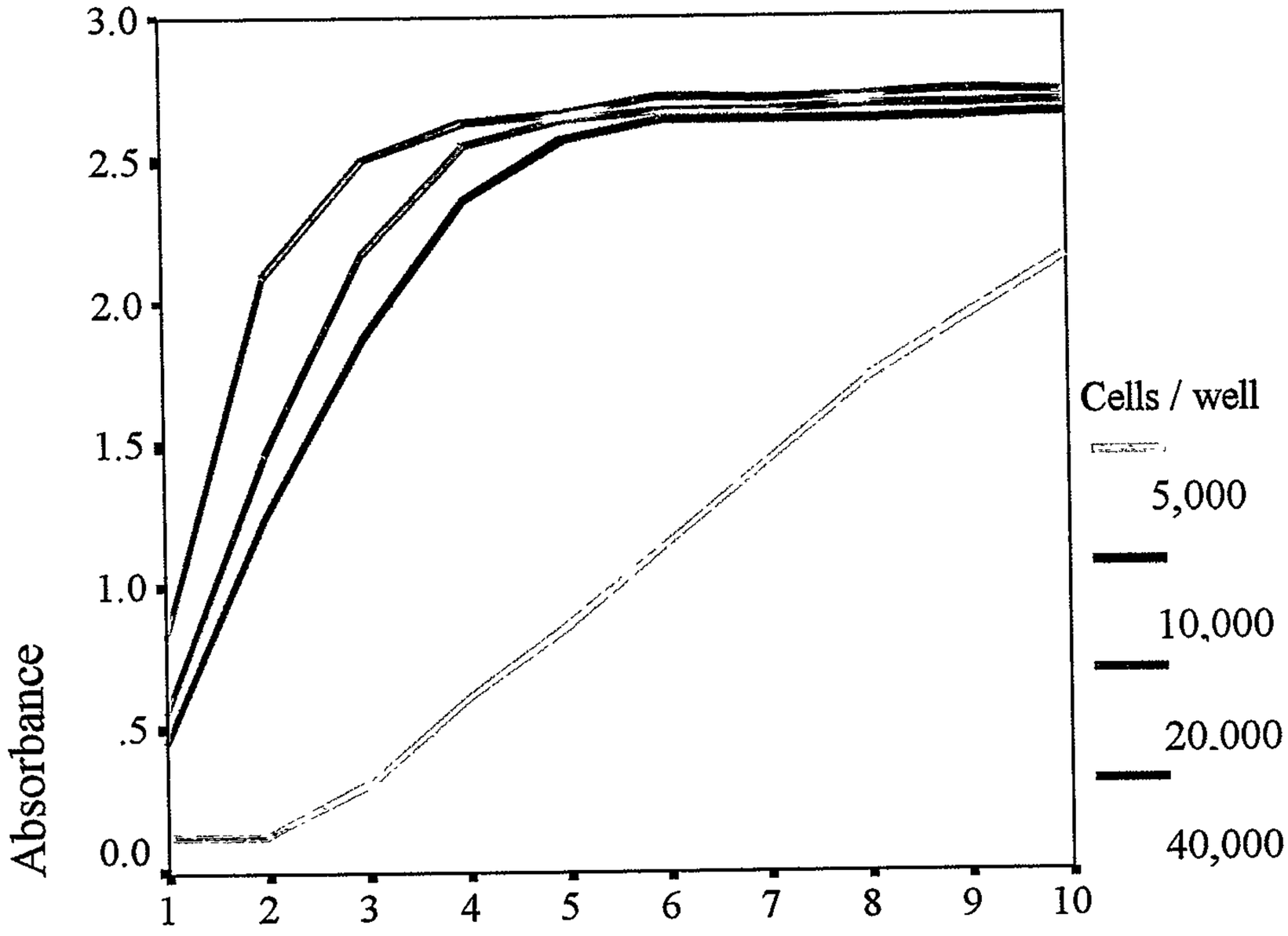

Figure 3.18. ALP assay results after 4 days of incubation

Figure 3.18 shows that the absorbance levelled off at 2.6. This was actually the maximum reading of the measurement of the spectrophotometer. It also showed that with 10,000 cells/well seeding density, the ALP activities were uniform in the first 3 minutes. The ALP activities were too fast with 20,000 cells/well seeding density. Seeding density with 10,000 cells/well appeared to be a suitable seeding density for the assay. To confirm the result, the ALP assay was performed on wells with different seeding densities after 7 days of incubation (Tab. 3.11 and Fig. 3.19)

Table 3.11. ALP assay results after 7 days of incubation

Time	5,000 cells/well	10,000 cells/well	15,000 cells/well	20,000 cells/well
1	1.036±0.103	1.560±0.215	2.287±0.095	2.123±0.204
2	1.788±0.182	2.341±0.161	2.586±0.040	2.550±0.101
3	2.331±0.159	2.583±0.126	2.602±0.041	2.553±0.067
4	2.530±0.065	2.622±0.103	2.601±0.036	2.542±0.060
5	2.612±0.085	2.625±0.099	2.590±0.036	2.531±0.061
6	2.623±0.080	2.621±0.107	2.590±0.037	2.528±0.064
7	2.627±0.086	2.614±0.091	2.587±0.043	2.516±0.064

Results from Table 3.11 and Figure 3.19 also showed that a seeding density of 10,000 cells/well would be appropriate for the ALP assay. The absorbance of the first three minutes would be used for the calculation of the rate of reaction.

3.4.3 Late effects of laser irradiation

Since a potential time window for observable changes of laser irradiation could not be identified in the pilot study, the following experiment was carried over a 9 day period. The ALP activities were analysed 24 hours after each irradiation for 7 days. “Day 1 plate” in the results indicated a plate being irradiated once. “Day 2 plate” represented a plate being irradiated twice. However, the actual incubation time was 2 days for “Day 1 plate” and 6 days for “Day 2 plate”. Seeding was done on “Day –1” and irradiation started 24 hours later on “Day 0”.

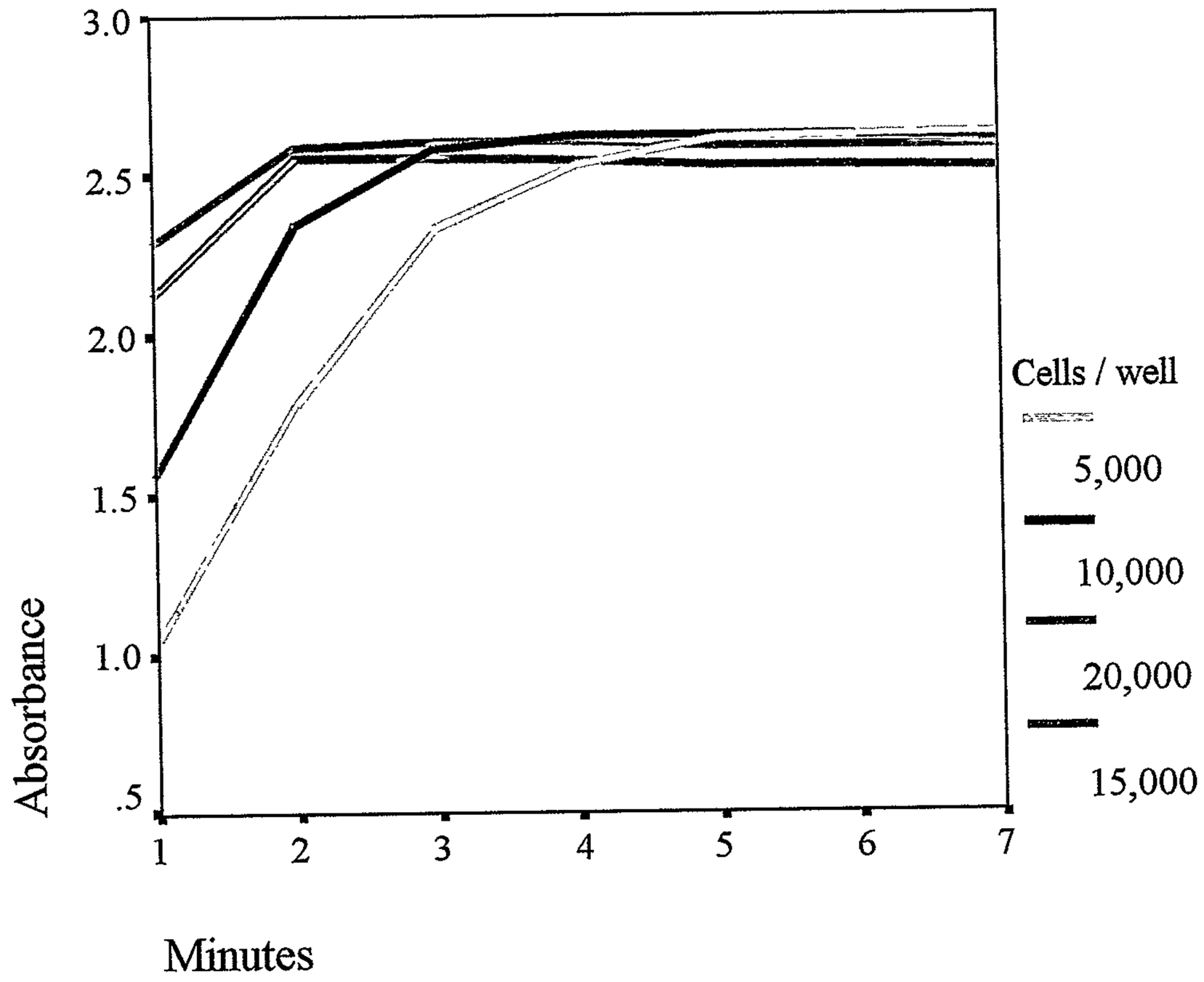

Figure 3.19. ALP assay results after 7 days of incubation

The linear changes of the absorbance of an individual well in the first few minutes were used to calculate the rate of reaction. It was calculated by linear regression. Figures 3.20 to 3.22 show the rate of reaction with days after 0.27J to 3.96J laser irradiations.

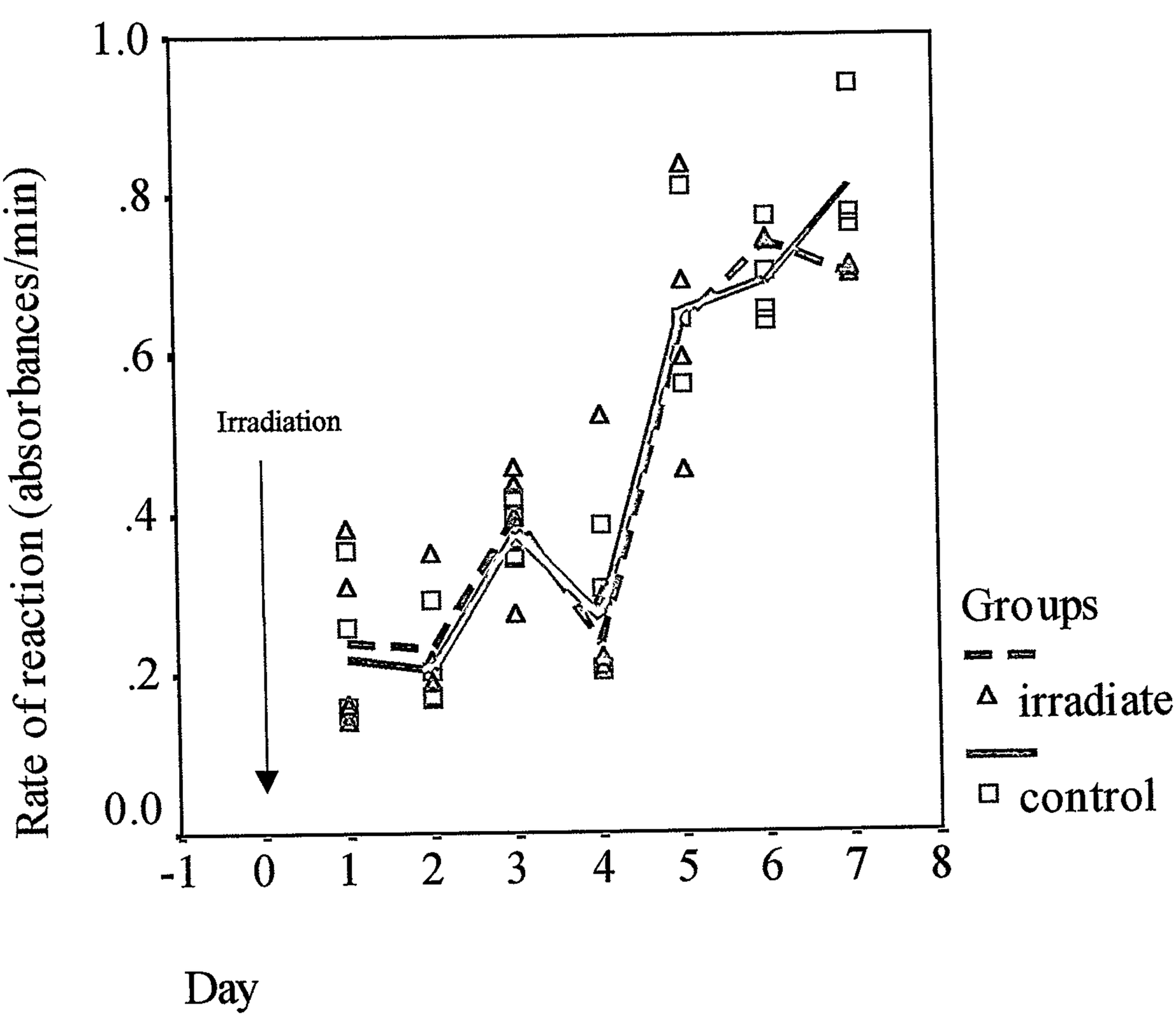

Figure 3.20. Rate of reaction after 0.27J irradiation (24 hours after)

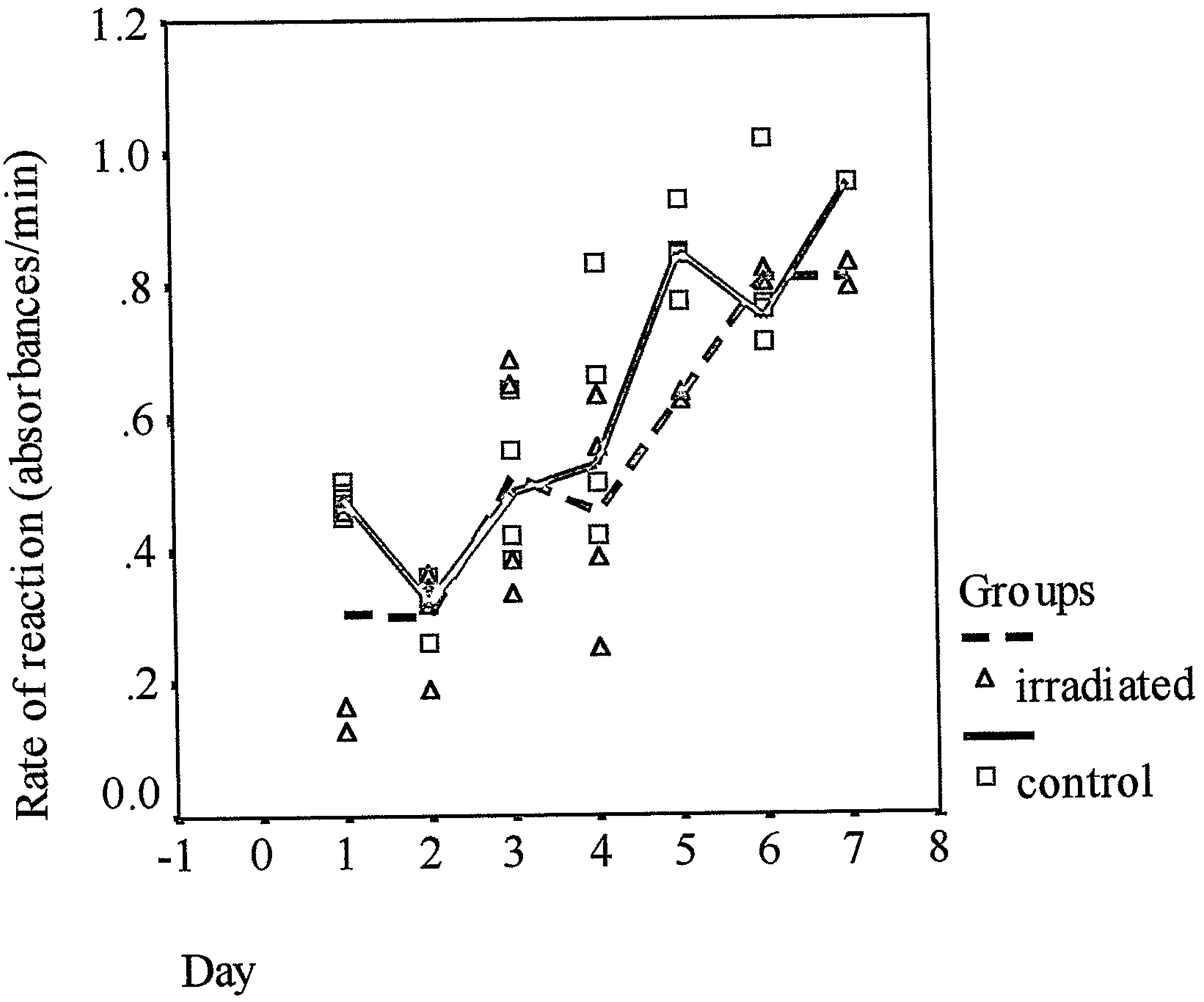

Figure 3.21. Rate of reaction after 0.99J irradiation (24 hours after)

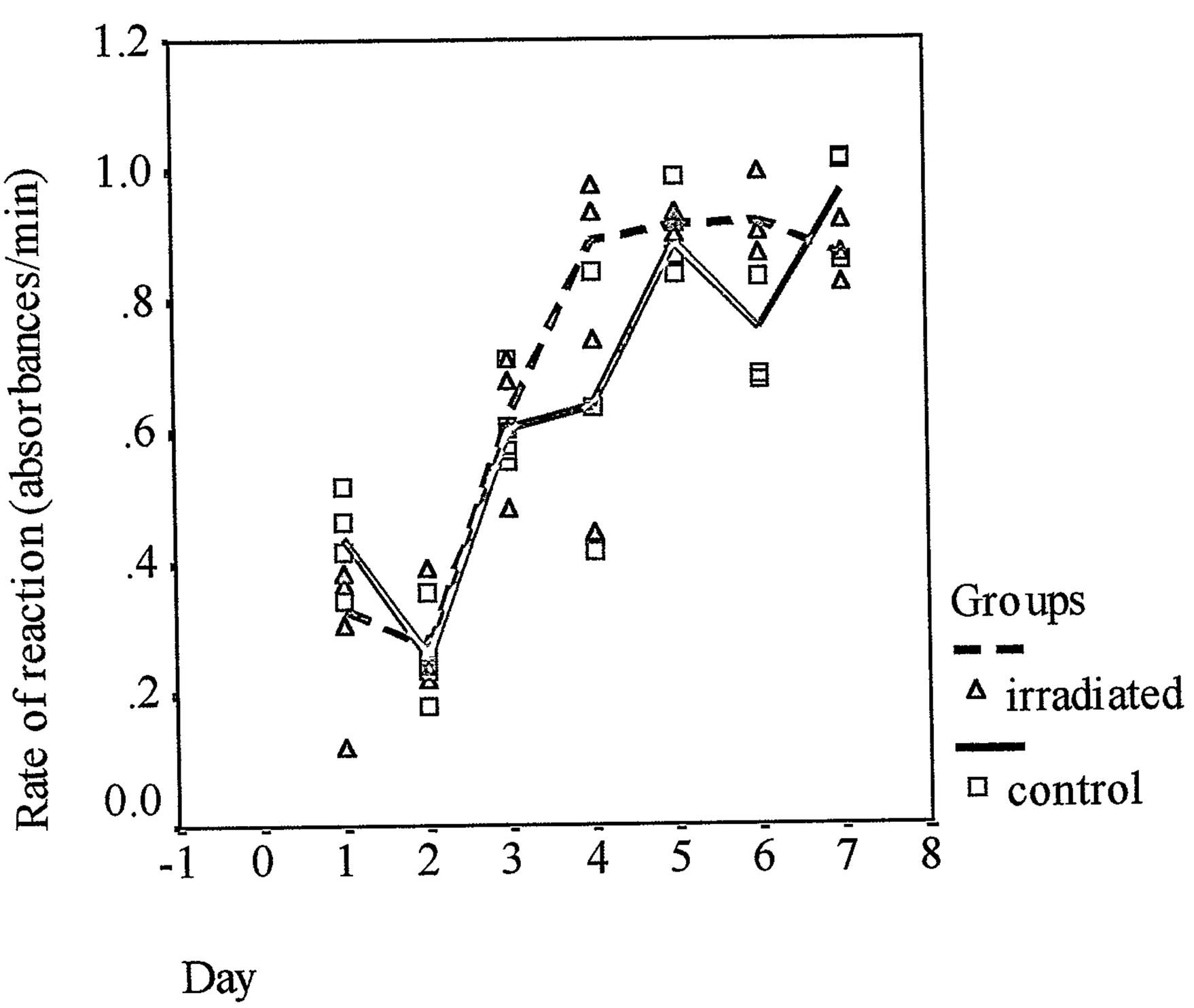

Figure 3.22. Rate of reaction after 3.96J irradiation (24 hours after)

The rate of reaction ie. ALP activities increased steadily with days in both irradiated and control groups with a tendency of decrease in ALP activities in the irradiated groups except with energy level 3.96J. The standard deviations of the results in both irradiated and control groups were large. Stimulation ratio was the ratio between the ALP activities of the irradiated group and those of the corresponding control group. Positive indicated an increase and negative indicated a decrease in ALP activities after laser irradiation. Figure 3.23 shows the stimulation ratio of the different energy levels.

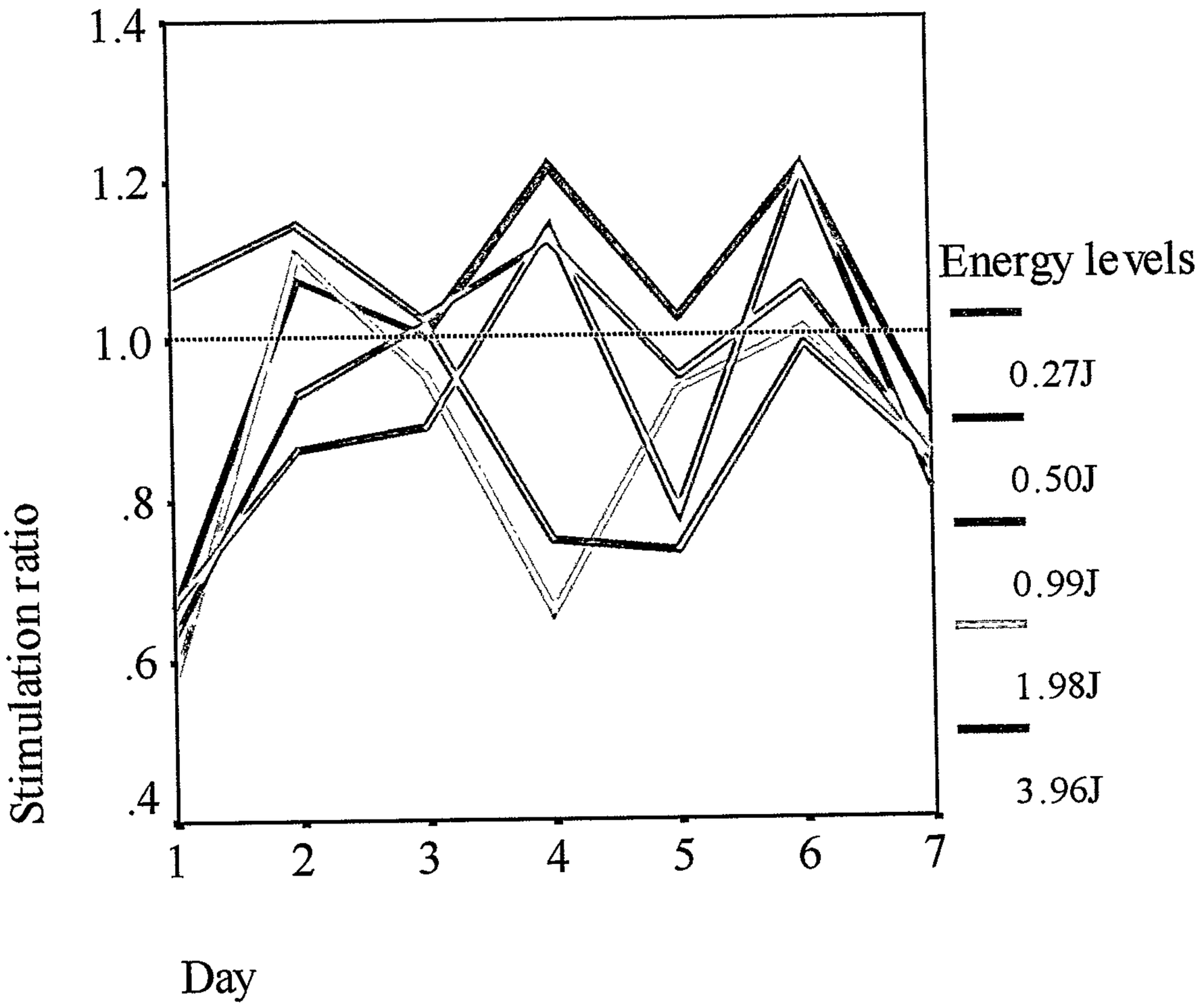

Figure 3.23. Stimulation ratio of different energy levels (24 hours after)

There was a trend that the laser irradiation had inhibitory effects. However, the fluctuation of the stimulation ratio did not follow a general pattern and the ratio was within the 10-20% range. The results were applied to independent t-test. Table 3.12 summarises the results of the late response in ALP activities with different energy levels.

Table 3.12. Summary of ALP assay: late effects

Energy level	Day	Control	SD (C)	Laser	SD (L)	p value	Stimulation ratio
0.27	1	0.230	0.096	0.246	0.117	0.839	1.070
0.27	2	0.210	0.058	0.241	0.073	0.535	1.147
0.27	3	0.378	0.038	0.389	0.083	0.824	1.028
0.27	4	0.276	0.088	0.310	0.147	0.706	1.124
0.27	5	0.673	0.127	0.642	0.161	0.787	0.954
0.27	6	0.693	0.059	0.739	0.003	0.214	1.067
0.27	7	0.825	0.098	0.702	0.006	0.159	0.850
0.50	1	0.466	0.076	0.313	0.180	0.191	0.671
0.50	2	0.286	0.067	0.248	0.069	0.451	0.865
0.50	3	0.511	0.098	0.456	0.196	0.637	0.891
0.50	4	0.523	0.209	0.596	0.194	0.659	1.140
0.50	5	0.801	0.010	0.627	0.008	0.003	0.783
0.50	6	0.608	0.122	0.737	0.085	0.356	1.212
0.50	7	0.915	0.033	0.744	0.002	0.086	0.813
0.99	1	0.479	0.024	0.303	0.184	0.152	0.632
0.99	2	0.321	0.043	0.299	0.078	0.651	0.933
0.99	3	0.499	0.118	0.510	0.179	0.926	1.021
0.99	4	0.604	0.182	0.453	0.169	0.271	0.750
0.99	5	0.848	0.064	0.626	0.009	0.005	0.738
0.99	6	0.815	0.138	0.810	0.013	0.945	0.994
0.99	7	0.953	0.001	0.809	0.025	0.079	0.849
1.98	1	0.492	0.056	0.284	0.192	0.116	0.577
1.98	2	0.334	0.076	0.371	0.123	0.634	1.109
1.98	3	0.603	0.100	0.578	0.156	0.796	0.958
1.98	4	0.807	0.051	0.532	0.208	0.304	0.660
1.98	5	0.878	0.037	0.822	0.119	0.626	0.936
1.98	6	0.768	0.090	0.778	0.047	0.868	1.013
1.98	7	0.939	0.162	0.806	0.023	0.288	0.858
3.96	1	0.437	0.074	0.291	0.122	0.098	0.666
3.96	2	0.259	0.072	0.279	0.076	0.719	1.076
3.96	3	0.611	0.071	0.615	0.102	0.951	1.007
3.96	4	0.632	0.213	0.770	0.241	0.461	1.218
3.96	5	0.891	0.067	0.915	0.016	0.531	1.027
3.96	6	0.759	0.089	0.923	0.064	0.036	1.217
3.96	7	0.963	0.090	0.870	0.048	0.210	0.903

There were three occasions that the differences between irradiated and control groups reached a statistically significant level (highlighted in Tab. 3.12). However, no trend was observed and dose related effects could not be found.

3.4.4 Early effects of laser irradiation

Results from the previous experiment showed that the ALP activities were high and the measurement frequently reached the machine limit near the end of the experimental period. A much lower seeding density (2,500 cells/well) was applied in the following experiments. Since the rate of reaction was relatively constant at the beginning of the reaction, only one measurement eg. absorbance at the end of 3 minutes was used to calculate the rate of reaction. The reaction was stopped with addition of 50μL of 1M NaOH. The experiment was carried out in a 7 days period. The ALP activities were analysed 1 hour after each irradiation for 5 days. The first irradiation and analysis were carried on the third day from seeding i.e. 48 hours of incubation. "Day 1 plate" in the results indicated a plate being irradiated once. Seeding was done on "Day –1" and irradiation started 48 hours later on" Day 1". Figures 3.24 to 3.26 show the rate of reaction with days after 0.27J to 3.96J laser irradiations.

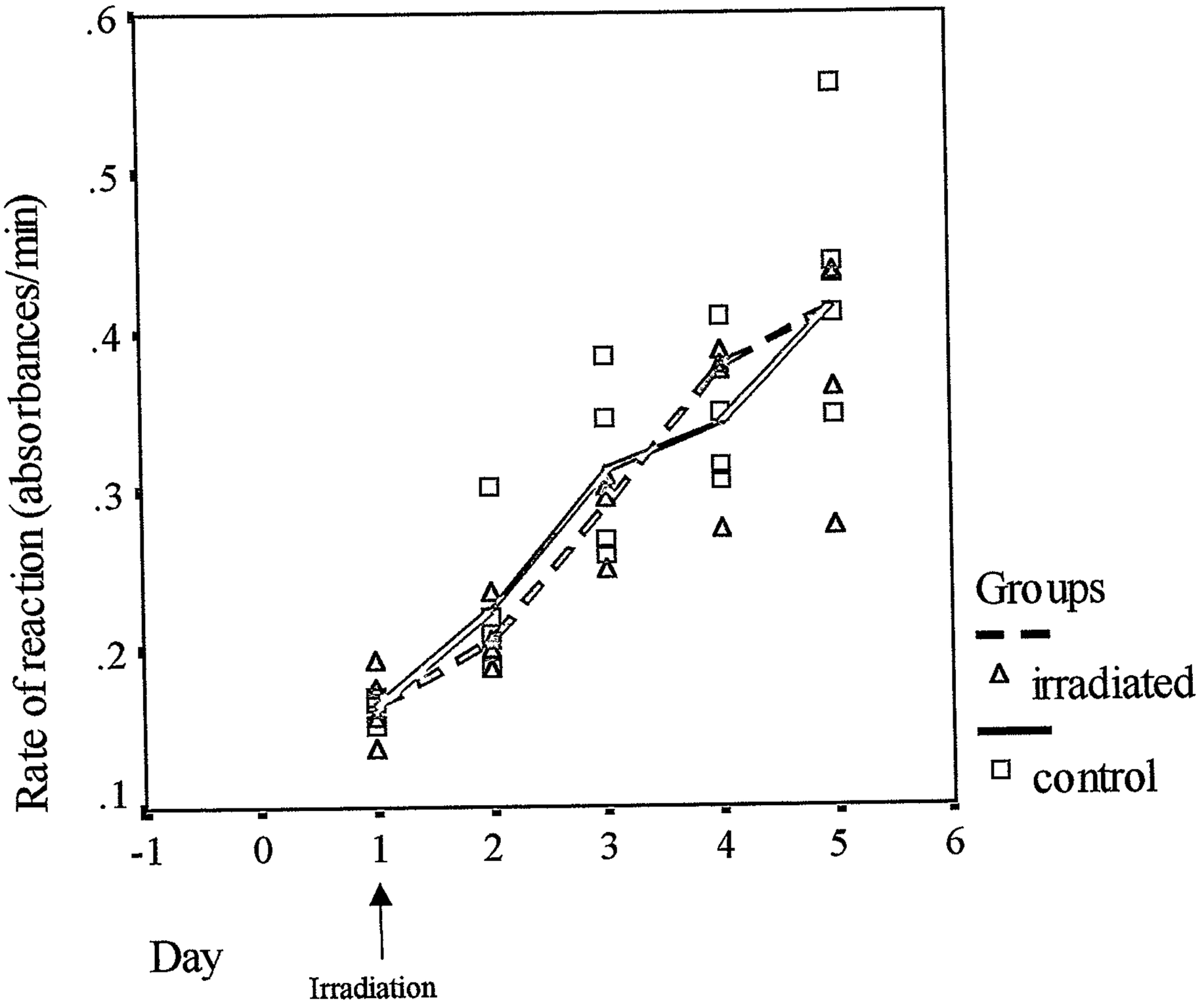

Figure 3.24. Rate of reaction after 0.27J irradiation (1 hour after)

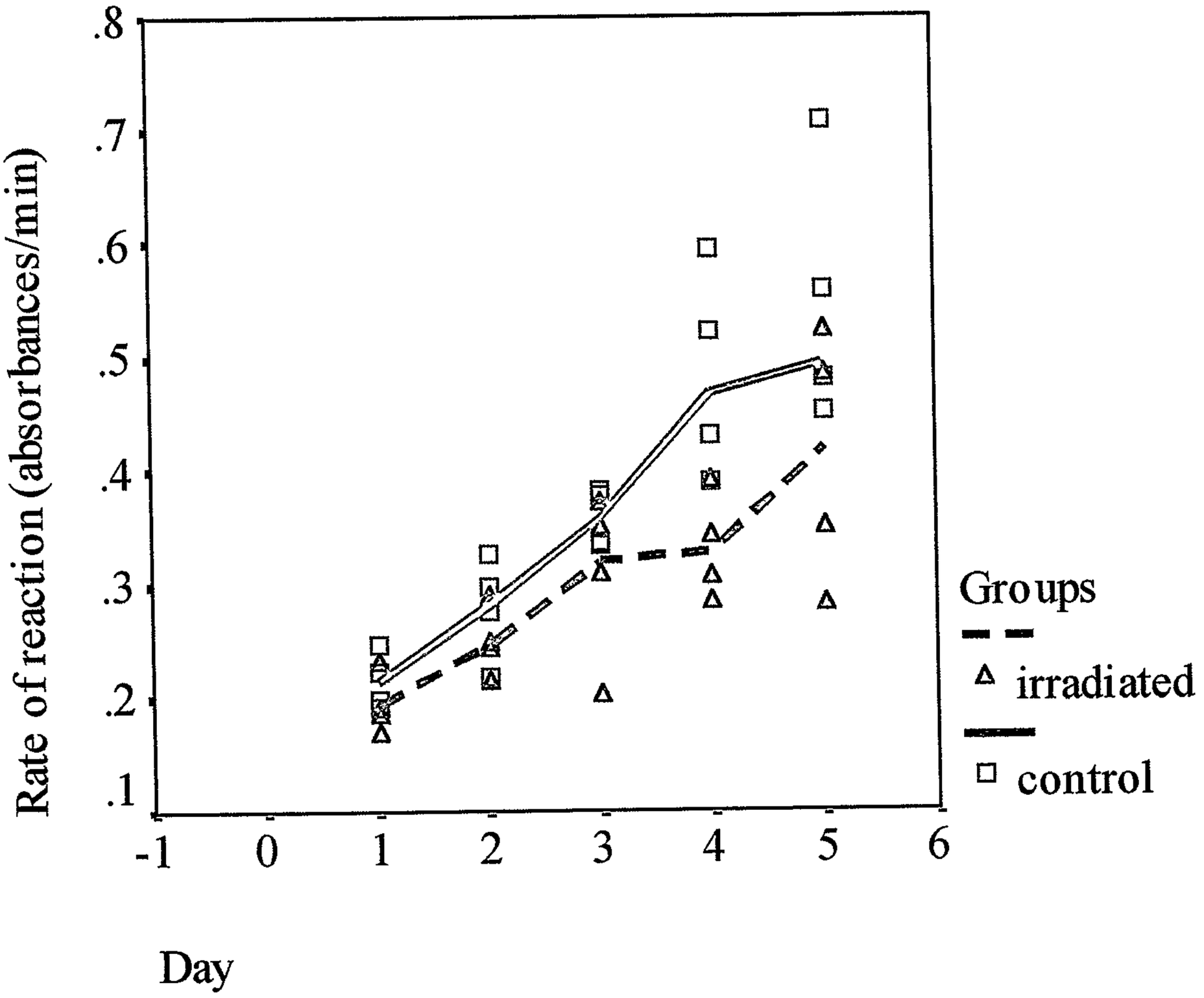

Figure 3.25. Rate of reaction after 0.99J irradiation (1 hour after)

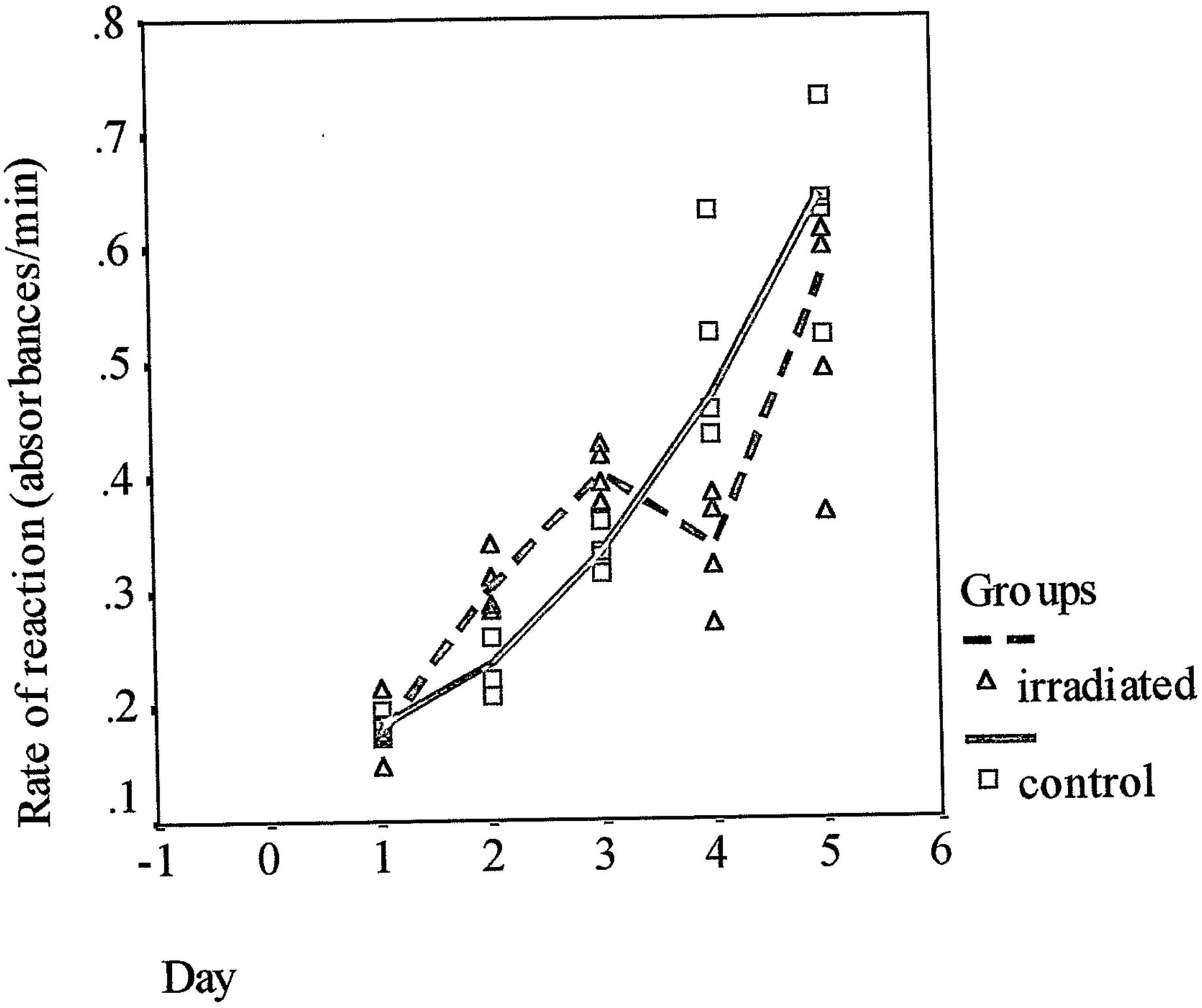

Figure 3.26. Rate of reaction after 3.96J irradiation (1 hour after)

The patterns of increase in rate of reaction were similar between irradiated and control groups. There was a tendency towards a decrease in ALP activities with laser irradiation after day 3 except with energy level 0.27J. However, the standard deviations in the irradiated and control groups were large. Figure 3.27 shows the stimulation ratio of different energy levels.

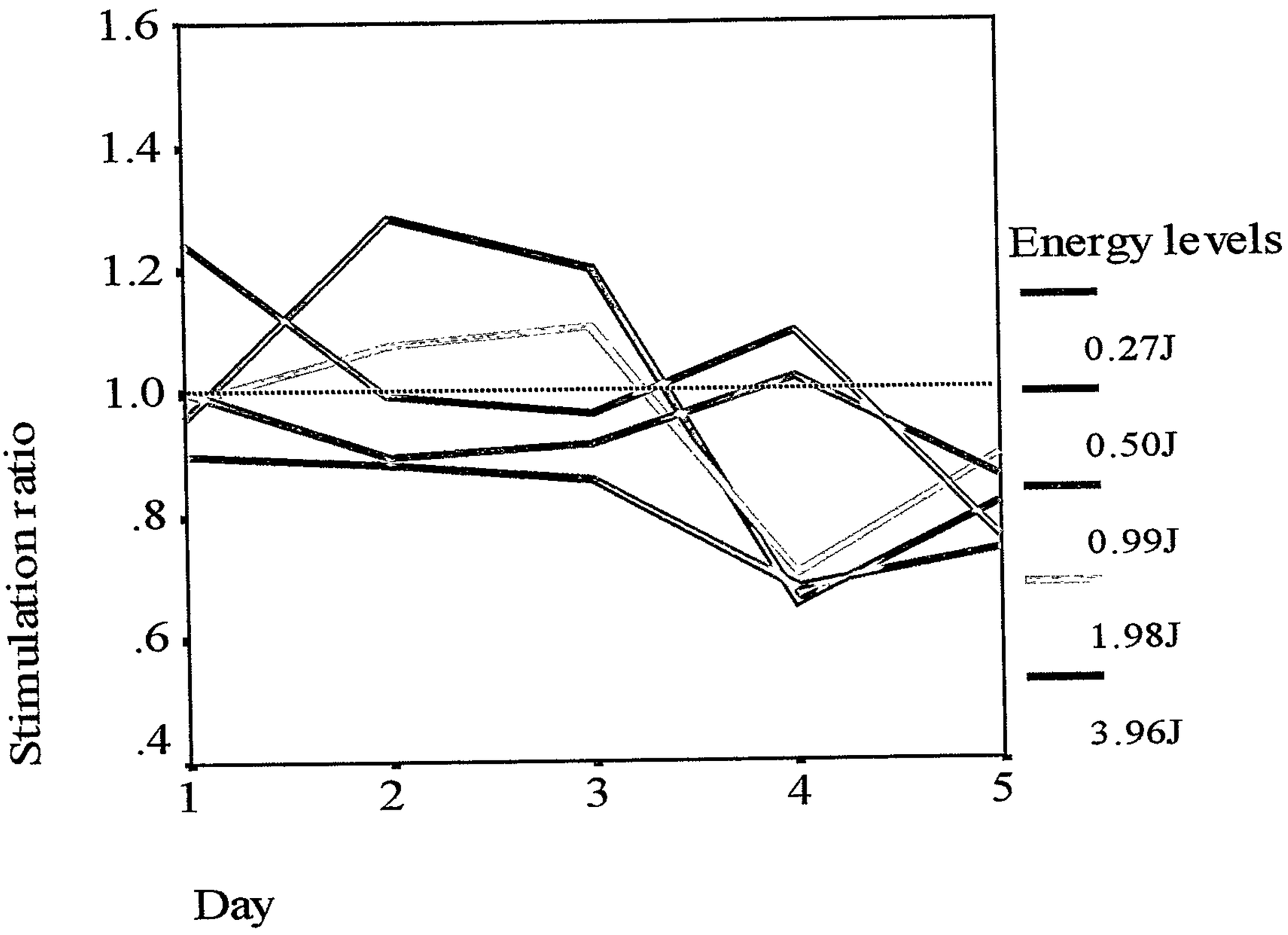

Figure 3.27. Stimulation ratio of different energy levels (1 hour after)

The lines of smaller energy levels (0.27J and 0.50J) had a different trend compared with those of higher energy levels (1.98J and 3.96J). It appeared that irradiation with smaller energy levels decreased ALP activities initially and then increased on day 4 and decreased again. Irradiation with larger energy levels produced the opposite effects. They increased ALP activities initially and decreased on day 4 and increased again on day 5. There was overall a stronger

tendency of inhibitory than stimulation effects. The fluctuation of the stimulation ratio was relatively small except for a large drop of stimulation ratio on Day 4. Table 3.13 summarises the results of the early response in ALP activities with different energy levels.

Table 3.13. Summary of ALP assay: early effects

Energy level	Day	Control	SD (C)	Laser	SD (L)	P value	Stimulation ratio
0.27	1	0.164	0.008	0.164	0.023	0.975	1.003
0.27	2	0.230	0.050	0.205	0.020	0.415	0.894
0.27	3	0.314	0.060	0.287	0.026	0.465	0.916
0.27	4	0.344	0.047	0.353	0.053	0.826	1.023
0.27	5	0.439	0.088	0.377	0.076	0.329	0.860
0.50	1	0.169	0.023	0.211	0.016	0.031	1.244
0.50	2	0.248	0.017	0.246	0.020	0.889	0.992
0.50	3	0.312	0.028	0.301	0.048	0.702	0.964
0.50	4	0.419	0.069	0.459	0.059	0.410	1.096
0.50	5	0.481	0.148	0.365	0.147	0.307	0.758
0.99	1	0.216	0.025	0.194	0.027	0.278	0.899
0.99	2	0.280	0.046	0.247	0.031	0.288	0.884
0.99	3	0.358	0.025	0.307	0.075	0.271	0.857
0.99	4	0.485	0.092	0.329	0.046	0.035	0.680
0.99	5	0.550	0.114	0.409	0.113	0.128	0.743
1.98	1	0.203	0.028	0.200	0.015	0.859	0.985
1.98	2	0.238	0.026	0.256	0.023	0.343	1.075
1.98	3	0.332	0.033	0.367	0.041	0.234	1.105
1.98	4	0.508	0.050	0.357	0.111	0.066	0.704
1.98	5	0.577	0.065	0.517	0.046	0.191	0.897
3.96	1	0.184	0.010	0.177	0.028	0.652	0.961
3.96	2	0.237	0.026	0.304	0.026	0.011	1.283
3.96	3	0.335	0.019	0.403	0.023	0.004	1.202
3.96	4	0.512	0.088	0.335	0.051	0.019	0.655
3.96	5	0.631	0.084	0.516	0.114	0.161	0.818

The initial increase in ALP activities with 0.50J laser irradiation on Day 1 and the decrease in ALP activities with 0.99J laser irradiation on Day 4 reached a statistically significant level. The increase and then decrease in ALP activities with 3.96J irradiation on Day 2 to Day 4 also reached a statistically significant level. It appeared that different energy levels produced different responses in ALP activities. However, the sample size was small (4 in each group per day). A more general pattern of dose related changes could not be determined.

3.4.5 Replicate of experiment: early effects

In order to further investigate the effects of the high energy level laser irradiation to ALP activities, experiments with energy levels: 0.99J, 1.98J and 3.96J and bigger sample size (6 in each group per day) were performed. Figures 3.27 to 3.29 show the changes of rate of reaction with days. The changes of rate of reaction were very similar between the irradiated and control groups. Figure 3.30 shows the stimulation ratio of the different energy levels. The changes of stimulation ratio were small in magnitude.

Table 3.14 summarises the results of the replicate. Although some of the results in the replicate reached a statistically significant level, they were not related to a clear trend.

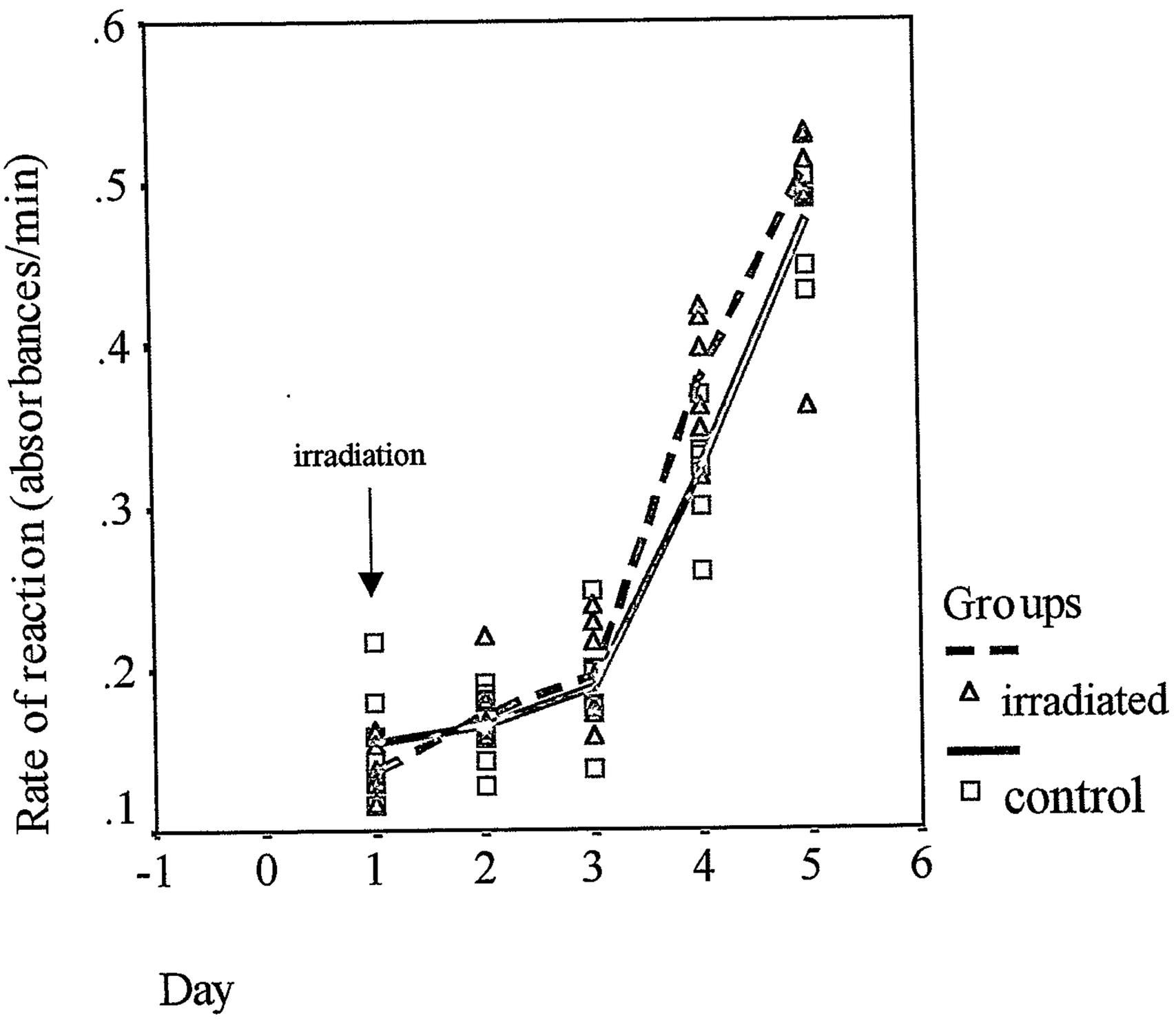

Figure 3.28. Rate of reaction after 0.99J irradiation (replicate: 1 hour after)

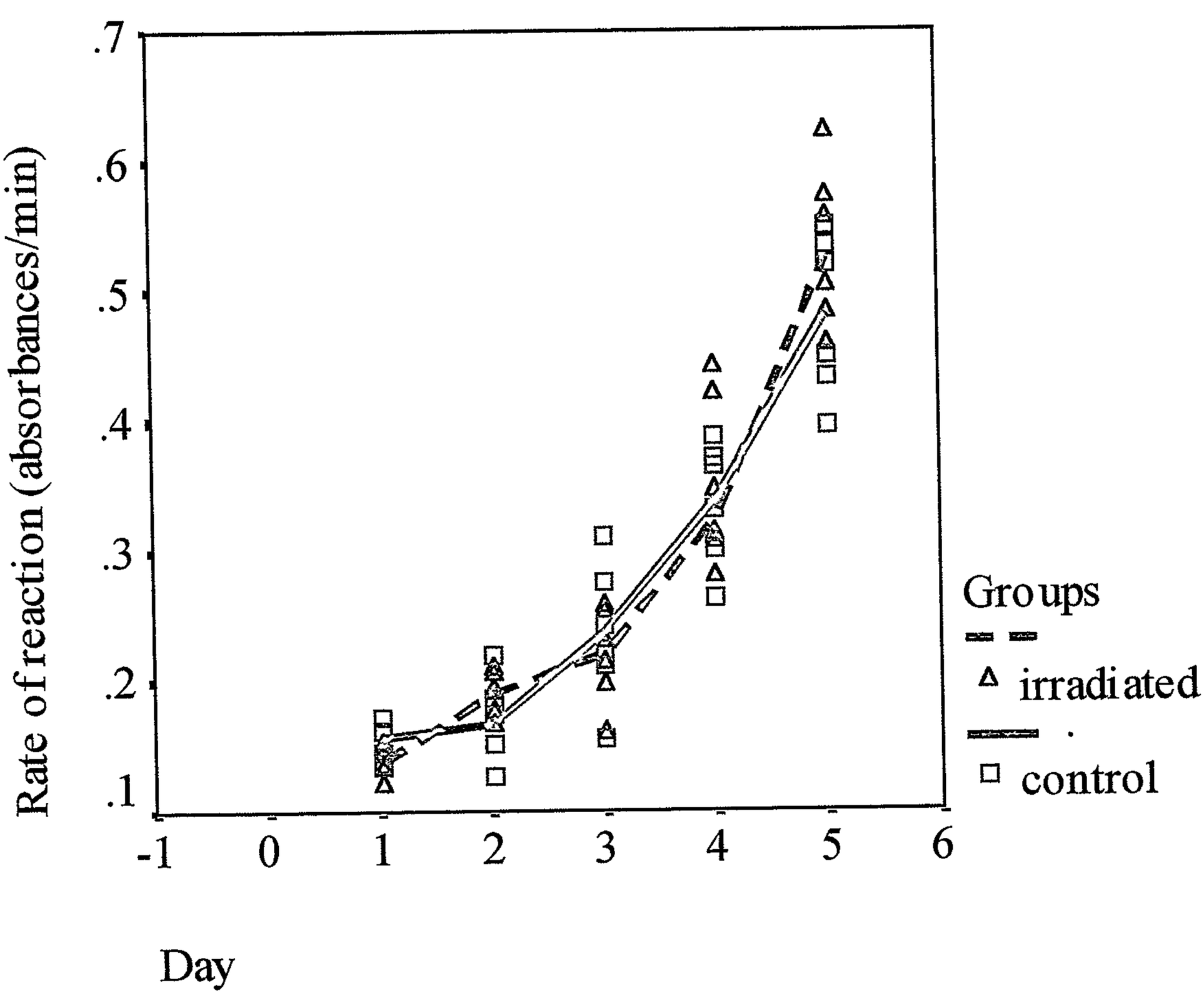

Figure 3.29. Rate of reaction after 1.98J irradiation (replicate: 1 hour after)

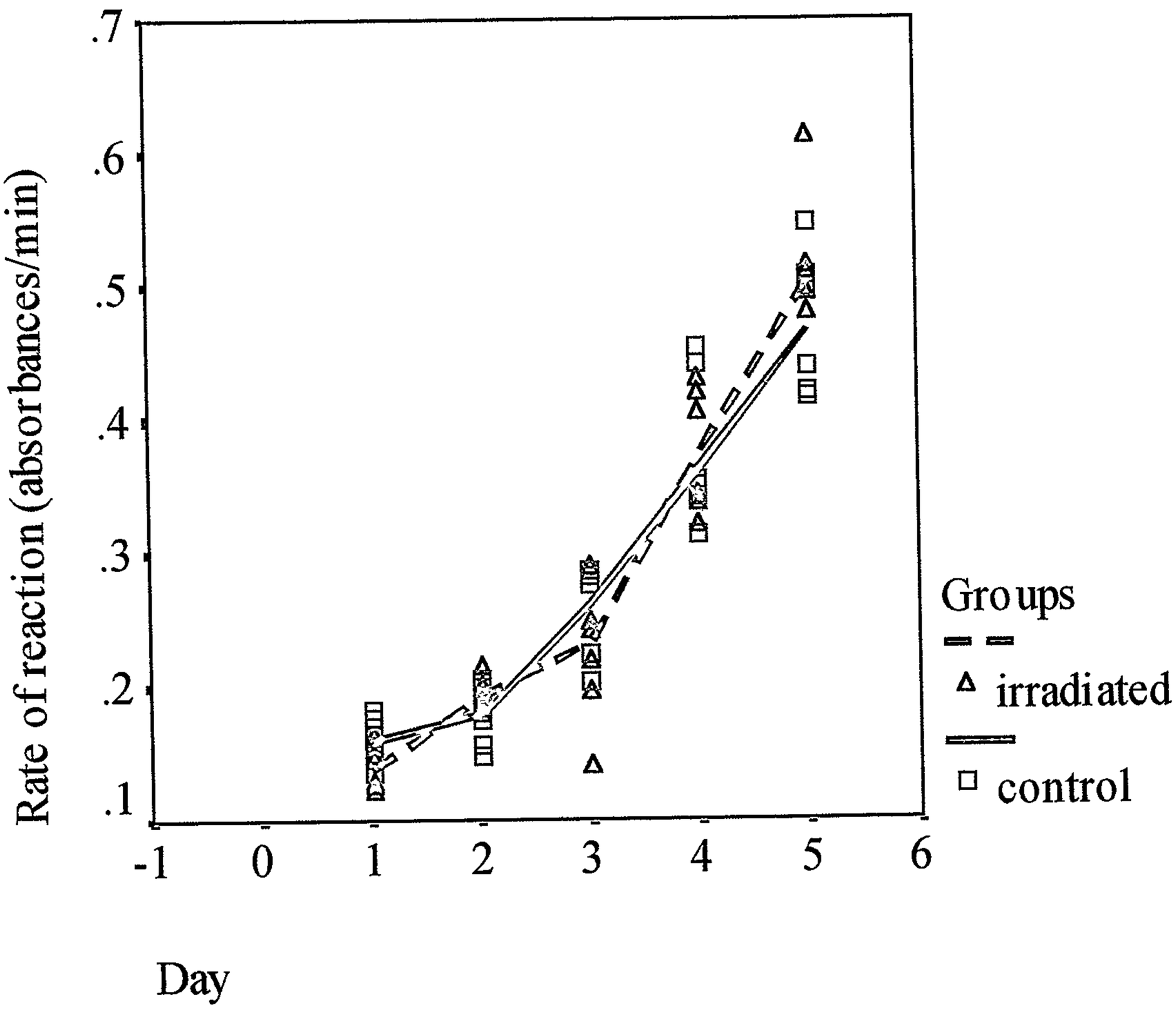

Figure 3.30. Rate of reaction after 3.96J irradiation (replicate: 1 hour after)

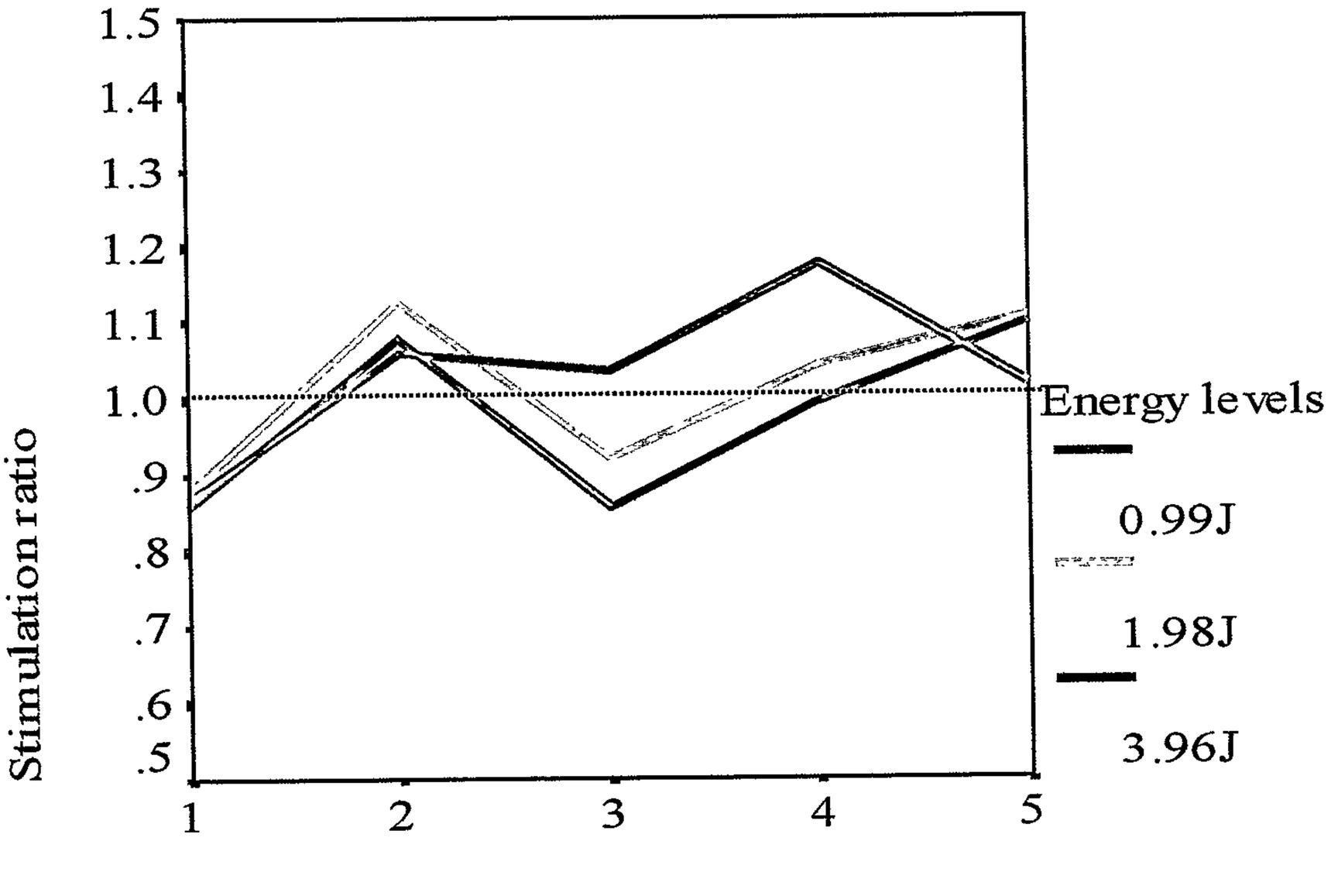

Figure 3.31. Stimulation ratio of different energy levels (rep1icate: 1 hour after)

Table 3.14. Summary of ALP assay: early effects (replicate)

Energy level	Day	Control	SD (C)	Laser	SD (L)	P value	Stimulation ratio
0.99	1	0.158	0.037	0.137	0.016	0.246	0.87
0.99	2	0.164	0.025	0.174	0.023	0.505	1.06
0.99	3	0.190	0.036	0.197	0.034	0.741	1.04
0.99	4	0.320	0.036	0.377	0.042	0.031	1.18
1.98	1	0.157	0.013	0.138	0.011	0.024	0.88
1.98	2	0.169	0.032	0.190	0.017	0.184	1.13
1.98	3	0.237	0.053	0.219	0.036	0.503	0.92
1.98	4	0.337	0.047	0.352	0.066	0.664	1.04
3.96	1	0.160	0.018	0.137	0.016	0.047	0.86
3.96	2	0.179	0.024	0.193	0.012	0.234	1.08
3.96	3	0.258	0.036	0.221	0.050	0.180	0.86
3.96	4	0.376	0.057	0.375	0.048	0.962	1.00

3.4.6 Dose-related response

The evidence of any dose related responses were analysed by plotting stimulation ratio against energy level from Day 1 to Day 5. Figures 3.32 to 3.34 show the dose related responses of ALP activities on Day 2, 3, and 4. Patterns of dose related response could not be identified.

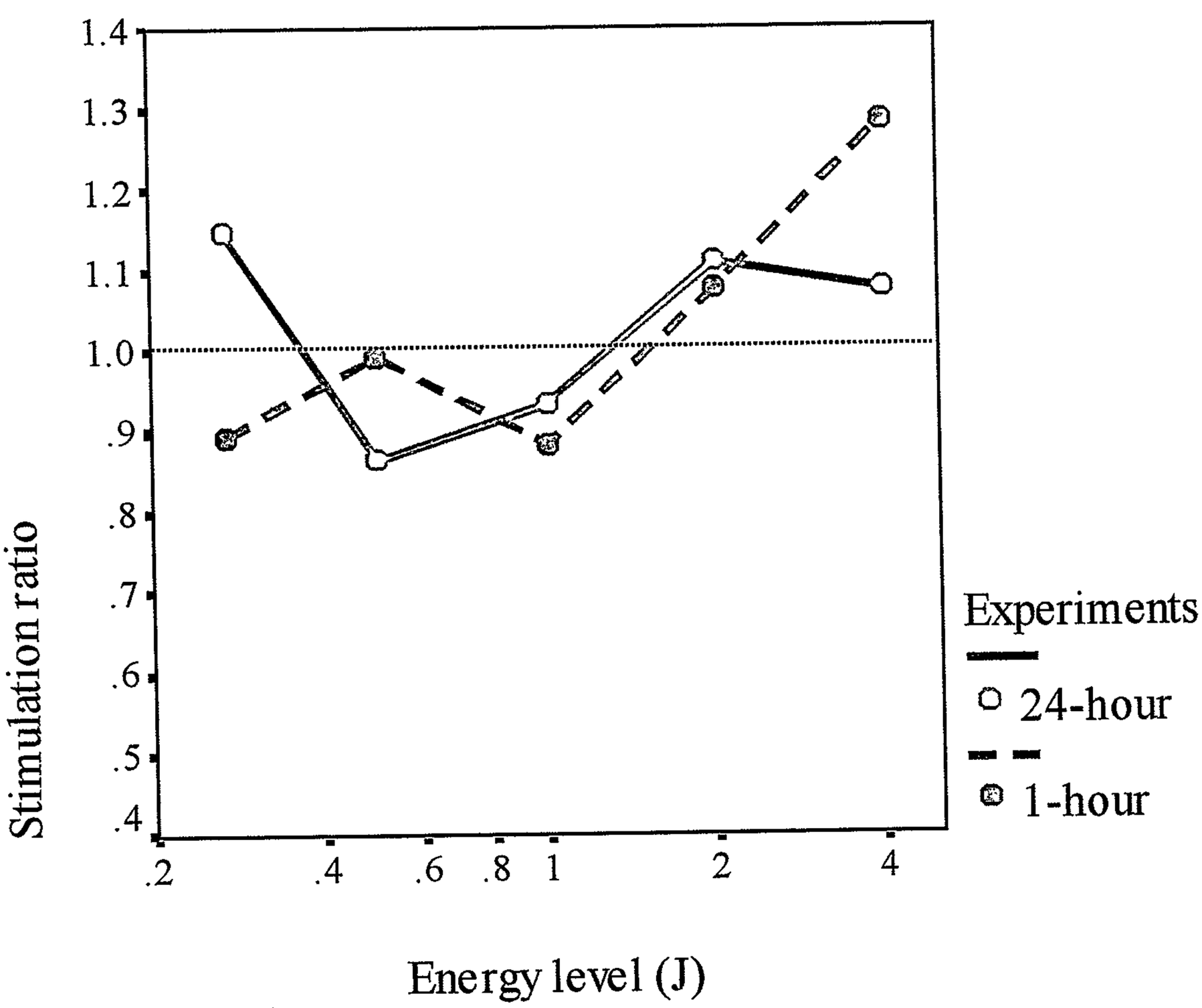

Figure 3.32. Day 2: dose related response

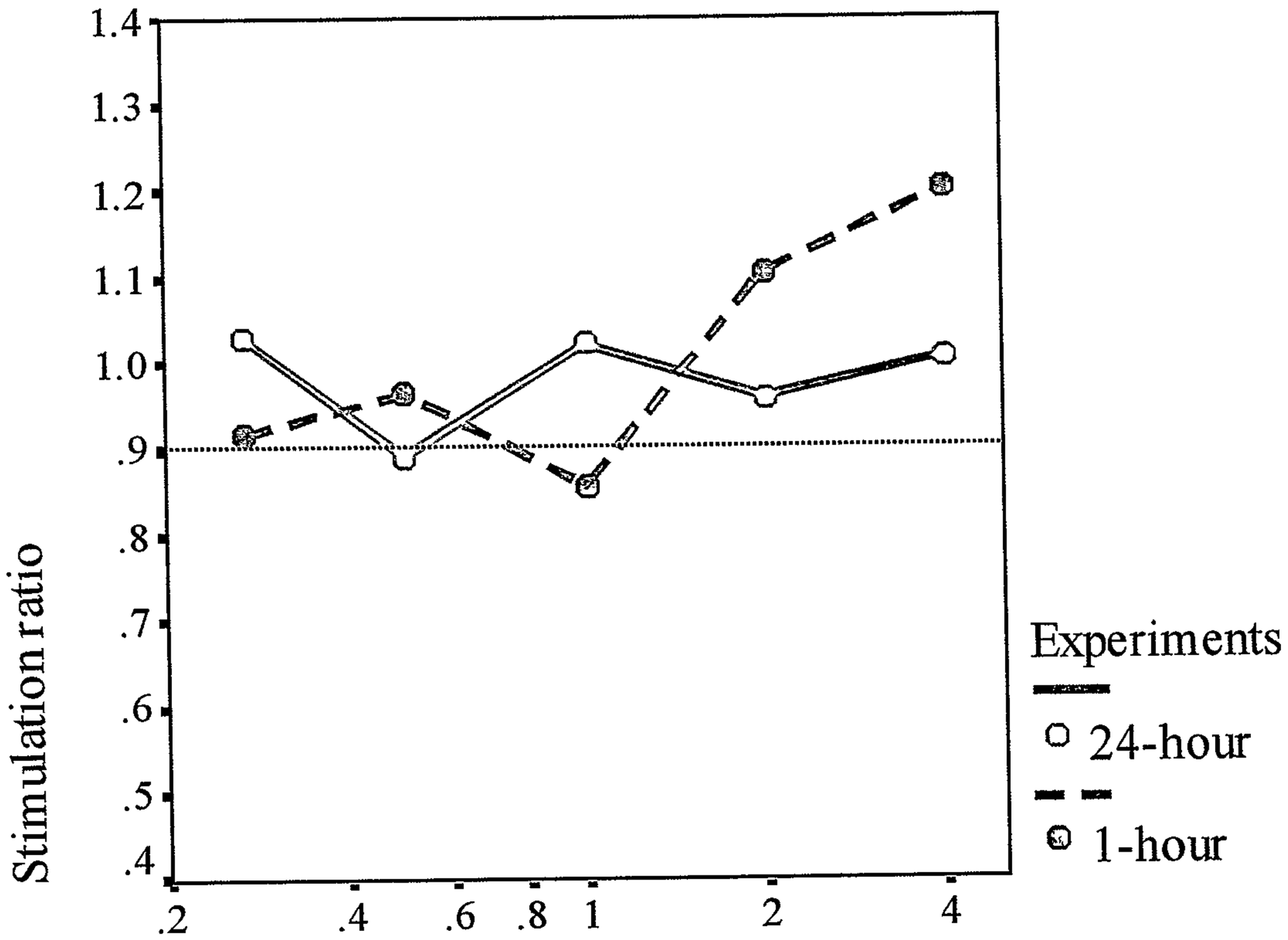

Figure 3.33. Day 3: dose related response

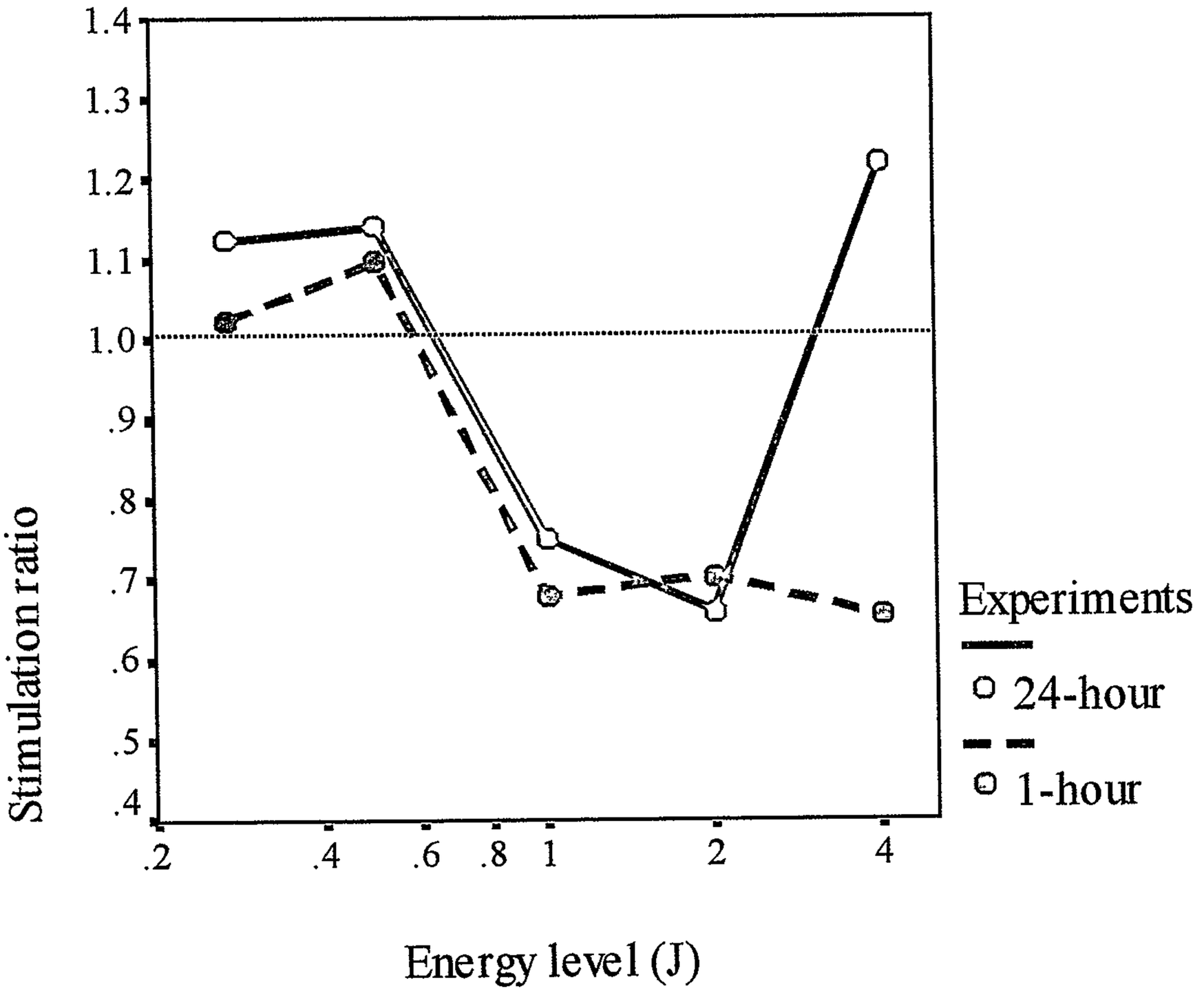

Figure 3.34. Day 4: dose related response

CHAPTER FOUR
DISCUSSION AND CONCLUSIONS

4.1 Laser equipment

The laser unit used in the experiment was called P-LaserSystem. It is a battery operated, cordless laser unit. The active laser medium is a semiconductor (diode) made of Gallium-Aluminium-Arsenide. The wavelength of the P-LaserSystem is 830nm. The beam which it generated was a true laser with the properties of monochromaticity, coherence and collimation. Most laser units in the past used solids or gases as the active laser medium eg. ruby laser and He-Ne laser. However, diode lasers have gained in popularity because of the cheaper cost in manufacturing, wavelength adjustment by changing the composition, and increase in output by clustering multiple diodes (Jenkins, personal communication 1998). Although most of the research in the last two decades has been done on the He-Ne laser (Trelles & Mayayo 1987, Hallman *et al.,* 1988, Pourreau-Schneider *et al.,* 1990, Lubart *et al.,* 1992a, Yamada 1991, Noble *et al.,* 1992, Rigau *et al.,* 1991, Ghamsari *et al.,* 1997, Harazaki & Isshiki 1997, Hashieh *et al.,* 1997, Harazaki *et al.,* 1998), there has been increasing interest in the diode laser, especially the diode with gallium and arsenide, (McCaughan *et al.,* 1985, Labbe *et al.,* 1990, Heussler *et al.,* 1993, Fernando *et al.,* 1993, Masse *et al.,* 1993, Roynesdal *et al.,* 1993, Khullar *et al.,* 1995, Lim *et al.,* 1995, El Sayed *et al.,* 1996, Khullar *et al.,* 1996, Skinner *et al.,* 1996, Akai *et al.,* 1997, Saito & Shimizu 1997, Usuba *et al.,* 1998).

The 9mm diameter optical tip was bigger than the internal diameter (6.4mm) and had the same dimension of the external diameter (9mm) of the well. It fitted nicely on the rim of the well. It provided a relatively constant reference point during irradiation. The distance between the cells and optical tip (depth of the well: 10.8mm) remained unchanged. The energy output of the laser was larger than what the manufacturer indicated. After the repair, the energy output increased even more. The actual energy output was measured by the power meter provided. The irradiation time was adjusted according.

Since the laser unit is designed for clinical use, it is not ideal for laboratory experiments. It is not robust enough for continuous use. The laser unit was broken twice during the period of the experiment. The unit was repaired in Denmark the first time. Then it was repaired locally in Sydney. Although the optical tip had a diameter of 9mm, the spot size was only 4.5mm in diameter. Each irradiation could only cover 15.9mm^2. The actual operating time of a fully charged battery was 30-40 minutes. Each well had to be irradiated separately. Only 4 samples of each energy level could be irradiated in a 7-day experiment. The sample size was therefore significantly limited by the above factors. In the later experiments (replicate: the early effects on ALP), shorter experimental period and fewer laser irradiated groups were used. Six samples of each energy level were irradiated. There was an improvement of the quality of the data.

4.2 Cell Culture

The human osteosarcoma cell line SAOS-2 was used in the present experiment because of its immortality and the osteoblastic properties. The responses of SAOS-2 to laser irradiation might give indications of the possible responses of the human osteoblasts to LLLT. This cell line has not previously been used in low level laser experiments. Human fibroblasts have been commonly used as the cell culture models in the recent studies (Labbe *et al.,* 1990, Loevschall & Arenholt-Bindslev 1994, Lubart *et al.,* 1992a, Pourreau-Schneider *et al.,* 1989, Pourreau-Schneider *et al.,* 1990, Rigau *et al.,* 1991, Hallman *et al.,* 1988). Loveschall & Arenholt-Bindslev (1994) used 35mm diameter petric dishes to seed the cells before irradiations. Labbe *et al.,* (1990) seeded the cells in 24-well culture clusters, which had a 1.88 cm^2 growth area. In both studies, the target area was much larger than the spot size of the laser. Loveschall & Arenholt-Bindslev (1994) used a special designed light expansion device which was not commercially available. Labbe *et al.,* (1990) increased the target-laser distance, so that the divergency of the laser fully irradiated the cells in a larger area. There was a substantial decrease in the energy density. However, laser beam should have minimal divergency. The latter method made dose calculation difficult.

Other cell lines have also been used in low level laser research such as mouse spleen cells (Karu *et al.,* 1989), mouse calvaria osteoblastic cells (Yamada 1991), bull sperms cells (Lubart *et al.,* 1992b), *E. coli* (Karu *et al.,* 1994a) and HeLa cells (Karu *et al.,* 1994b, Karu *et al.,* 1995). Studies using osteoblastic cell line are most relevant to orthodontia. Yamada (1991) concluded that laser irradiation only had more profound effects during the growing phase of the mouse osteoblastic cells. Therefore, the current study used osteosarcoma cells which were immortal and retained the osteoblastic characteristics (Rodan *et al.,* 1987). Rodan *et al.,* (1987) suggested that the SAOS-2 cells possessed several osteoblastic features including high ALP reaction, and could be useful as a permanent line of human osteoblast-like cells and as a source of bone-related molecules.

4.3 Seeding density

Much effort was put in to identify optimal seeding density for the different experiments and assays. A seeding density of 10,000 cells/well (10,000 cells/200μL, 50,000 cells/mL) was used for the MTT assay in the pilot study and 20,000 cells/mL (20,000 cells/200μL, 10,000 cells/mL) was used for SDS-Page. Seeding densities of 10,000 cells/well (10,000 cells/200μL, 50,000 cells/mL) and subsequently 2,500 cells/well (2500 cells/200μL, 12500 cells/mL) were used for ALP assay. The 10,000 cells/well seeding density was higher than that used by Farley *et al.,* (1994). Farley *et al.,* (1994) seeded SAOS-2 in a 24 well plate with 0.5mL /well (growth area: 1.88 cm^2) of 35,000 cells/mL aliquots for cell surface ALP activities assay. Ten thousand cells/well was equivalent to 31,250 cells/cm^2 in a 96-well plate while 12,500 cells/well was equivalent to 9,300 cells/cm^2 in a 24-well plate. (Farley *et al.,* 1994). The 2,500 cells/well or 7,800 cells/cm^2 seeding density was closer to which they suggested for ALP activities assay. Yamada (1991) also seeded 7,890 cells/cm^2 of clonal osteoblastic cells in a 12-well plate for the ALP activity assay. The choice of higher seeding density initially was due to concern of a possible decrease in ALP activities after laser irradiation and relatively small number of cells in the well. However, it was found that a lower seeding density produced better data for ALP.

4.4 Irradiation procedure

The cells were irradiated with 3 to 5 different energy levels. The energy levels were 0.28J to 4.2J in the pilot study and 0.27J to 3.96J in the following experiments. The energy levels were calculated according to the energy output and intervals of half a second. The different energy intensities covered the common range of energy intensities recommended in biostimulation studies and in clinical use (Basford 1995, Walsh 1997a, Kert & Rose 1989, Karu 1998). The cells were irradiated daily. Multiple irradiations are recommended by most authors. A black plastic board at the bottom of the plate was used to minimise the reflection and scattering of the laser beam. Since it took a rather long time to irradiate a whole plate (more than 6 minutes), an individual plate was only partially irradiated at one time. Then the plate was irradiated again after 4 minutes (after partial irradiations of another two plates). This minimised the time a plate was out of the incubator. The time of irradiation was signalled by a peeping sound every half a second. Although there was a timer attached to the battery pack, it only counted a pre-determinied interval ie. 15 seconds, 30 seconds, 60 seconds or 120 seconds.

4.5 Time window for observable changes

In order to optimise the number of samples, it was important to estimate the time window for observable changes and narrow the range of energy levels applied. After determining the seeding density, the cells were irradiated with different energy levels for multiple intervals. Skinner *et al.,* (1996) found that there was a maximum increase in collagen production and cell biostimulation after 4 episodes of laser treatment. Loevschall *et al.,* (1994) examined morphology, cell number and cell viability on days 4 and 6 in their experiment. Pourreau-Schneider *et al.,* (1989) also found an increase in fibroblast numbers on day 4 after a single dose of laser irradiation (wavelength: 032.8nm, total dose: 1.2J/cm^2) and with further increase on day 7 with multiple irradiations. The time window for observable changes could not be determined in the pilot study. While Hallman *et al.,* (1988) could not found any effects of daily laser irradiation (wavelength: 633nm, power intensity: 24.7mW/cm^2) on fibroblast proliferation, their experiment was run for a 5 days period. It was decided that the period for experiments should be from 5 to

9 days for the present study. Observations were made either daily or every two days (after every laser irradiation or every two irradiations)

4.6 Protein expression

A previous study showed an increase in DNA synthesis after laser irradiation (Loevschall *et al.,* 1994). An increase in hydroxyproline formation was also found (Labbe *et al.,* 1990). In order to investigate the possible effects of laser irradiation on protein expression, SAOS-2 cells were irradiated with 3 different energy levels and analysed after 2 and 4 irradiations. The mini-gels of 2 daily irradiation were too faint to be scanned. Bands could be observed in the gels by eye. However after scanning, the bands did not have enough contrast. It was probably due to insufficient cells in a small well. Plates with bigger wells such as 8 and 24-well plates were tried for the experiment. However, it was difficult to irradiate such a large target area. The mini-gels of 4 daily irradiations gave good contrast of the bands. Compared with standards (Bio-Rad, low range), contamination of the results from foetal calf serum in the medium was unlikely. The assay was a screening assay to identify the region of changes. Coomassie staining gave quantitative data while silver staining which was a ultra sensitive protein stain showed qualitative results of changes of protein expression after multiple laser irradiations. The assay examined the global changes of protein expression. A more specific assay may be employed to find out more details of the possible proteins involved when the regions of interest are identified. Since a late or accumulative response (after multiple irradiations) was suspected according to the literature, the electrophoresis was performed 24 hours after the last irradiation. The experiment was then repeated to investigate any possible early effects of laser irradiation. The present study could not find any observable late or early changes. The differences between irradiated and control groups were not significant. The graphs of superimposition indicated that the peaks of both groups followed very similar pattern and similar in densities. In some pairs, the two graphs were almost superimposable.

Skinner *et al.,* (1996), using assay following pepsin digestion of cell cultures, found that there was an increase in secretion of procollagen by embryonic

fibroblasts after multiple laser irradiations. He commented that single or repeated irradiation at 24 hour intervals did not have a pronounced effect of collagen synthesis. However, extension of the laser schedule over 3 or 4 consecutive days did produce significant results (Skinner *et al.,* 1996). Hernandez *et al.,* (1989) reported signs of cellular hypertrophy including enlargement and dilatation of rough endoplasmic reticulum and Golgi apparatus in rats thyroid cells after thyroid glands were irradiated with a diode laser at a wavelength of 904nm and energy levels 10J to 60J. The authors also claimed a dose-related response. However, the authors did not indicate the criteria for measuring cellular hypertrophy and did not detail the other parameters of irradiation i.e. spot size of laser beam, average energy output, exposure regime.

Yamada (1991) also revealed a tendency for enlargement of Golgi apparatus detected by electron microscopy of laser treated clonal osteoblastic cells, indicating high secretory activities. However, biochemical examination showed no increase in the protein content of the laser-treated cells. Moreover, the author observed no measurable morphological difference between the irradiated and control cells (Yamada 1991). The cell line used in the present study was osteoblastic which was similar to the cell line used in Yamada's study. The result of the present study also agreed with this part of his finding.

4.7 ALP activities

Animal studies suggested that after tooth extraction and midpalatal expansion in rats, laser irradiation improved bone healing (Takeda 1988, Saito & Shimizu 1997). The results may or may not apply to a human model. The human osteosarcoma cell line, SAOS-2, produces skeletal type ALP isoenzyme which is also a product of osteoblasts (Farley *et al.,* 1989). The effects of laser irradiation on ALP activities may therefore give some indication of effects of laser irradiation on bone formation.

ALP activity is measured by the amount of p-nitrophenol liberated per minute (Rej *et al.,* 1981) or the amount of p-nitrophenol per minute per mg of protein (Farely *et al.,* 1991). In the present study, a standard curve with known

concentration of p-nitrophenol and a total volume of 100 μL was established. It was found that the concentration of nitrophenol was directly proportional to the measurement of absorbances at 405nm with the interception close to zero. The absorbance was directly read from the 96 wells plate and the amount of protein in the well was not measured. Moreover, the ALP activity was only for comparative purposes irradiated and control groups. Therefore, the relative ALP activities were expressed as absorbance changes per minute.

Time-dependent increases in optical density at 405nm were measured on a spectrophotometer (Farley *et al.,* 1991). The initial phase of the increase was linear. Analysis with regression gave the rate of changes per minute. Since the ALP activities were very high, the plates were chilled on ice before the phosphatase substrate was added. Delay in transferring the plates to the spectrophotometer could affect the results. Since it was found that the increase of absorbances in the first 3 minutes was linear, a single measurement at 3 minutes was used to calculate the ALP activity. The reaction was stopped by adding 50μL of 1M NaOH. Moreover, the seeding density was decreased to 2,500 cells/well. The plates were kept in the incubator during most of the 3 minute periods. The advantages of the latter approach included better temperature control, more accurate time control and fewer measurements. The overall results of the late (24 hours) and early (1 hour) effects showed that the standard deviations of the irradiated and the control groups were large. There was a tendency for a decrease in ALP activity with an increase in energy level. Some of the differences between irradiated and control groups reached a statistically significant level. However, no conclusive results could be obtained. Moreover, the replicate of early effects of laser irradiation with higher energy levels did not confirm the findings that laser irradiation with 3.96J decreased ALP activities significantly. The stimulation ratios fluctuated within a narrow range in most circumstances. By comparing the energy levels to the stimulation ratios by day, no pattern of dose-related response of ALP activities could be constructed. Yamada (1991) observed the same results in a laser study with mouse osteoblastic cells. Although he reported that ^{45}Ca accumulation in the cells and matrix layer was enhanced by laser irradiation by laser irradiation at $1J/cm^2$, there was no significant increase in ALP activities produced by laser irradiation.

The negative results obtained in the present study were partly related to the small sample size and large standard deviations. The sample size of the replicate was increased by 50% and the variance was smaller. However, the replicate failed to support any significant effects of laser irradiation on ALP activities in SAOS-2. Secondly, SAOS-2 had very high ALP activities ie. 100-1000 times higher than that of two other human osteosarcoma cell lines (TE-85 and SAOS-1) (Murray *et al.,* 1987). Yamada (1991) suggested that due to the marked increase in basal ALP activities in control cultures, the effects of laser irradiation on ALP activities could not be demonstrated. Moreover, there were two subpopulations of the human osteosarcoma cell line SAOS-2 (Farley *et al.,* 1991). The two phenotypes had different ALP activities: high ALP activities and low ALP activities ranging from 0.05U/mg protein to 2.5U/mg protein. A heterogeneous culture might have different response to laser irradiation, affecting the overall results (Karu 1998). In the present study, there were no significant late or early effects of multiple laser irradiations with different energy level on ALP activities in SAOS-2.

4.8 Controversies and limitations

Reviews from several authors (Basford 1986, Midda & Renton-Harper 1991, Basford 1995, Walsh 1997a, Walsh 1997b) suggested that there are still many unanswered questions in LLLT. Midda & Renton-Harper (1991) stated in their review articles that even after 20 years, the role of laser biostimulation in wound healing had not become established. Eight years later, although much work has been done, the inconsistency of the results remains a major challenge.

Karu (1998) suggested that even when the same culture of cells is irradiated, the degree of biological response can differ. One key event among the secondary reactions of cellular responses such as cell proliferation is the change in overall redox state of the irradiated cell. The overall redox state represents the net balance between stable and unstable reducing and oxidizing equivalents in dynamic equilibrium. The cellular response is weak or absent when the overall redox potential of a cell is optimal or near optimal for the particular growth conditions, and stronger when the redox potential of the target cell is initially shifted to a

more reduced state. This explains why the degrees of cellular responses can differ markedly in different experiments, and can sometimes be non-existent.

An irradiation-induced alteration in the overall cellular redox state is considered a key step in secondary reactions, but it does not explain all irradiation effects. It is shown that more than one function of a cell can be influenced by irradiation. Examples are, proliferation of fibroblasts (Loevschall & Arenholt-Bindslev 1994), increased motility of fibroblasts (Noble *et al.*, 1992) and collagen synthesis in fibroblasts (Skinner *et al.*, 1996). Analysis of this data clearly showed that when one cellular function was altered by irradiation, the others remained unchanged (Karu 1998). A possible explanation could be as follows. After laser irradiation, a jump in the intracellular pH (pH_i) has been detected. pH_i is a parameter of cellular homeostasis which is closely connected with cellular redox state. Stimulation of *E. coli* growth by monochromatic visible light has been suggested to be a pH-dependent process controlled at the level of gene expression (Karu 1998). However, growth stimulation is not the only process utilising the pH gradient. It has been proposed that there is a priority in the utilisation of pH gradient for ATP synthesis, nutrient transport, taxism and initiation of transcription. This priority enables the metabolic processes utilising the pH gradient to be regulated and coordinated (Karu 1998). However, how the priority between motility, secretory and replicative functions in fibroblasts is regulated to enable a cell to respond in a certain way, is not yet understood.

The two most important areas of low level laser therapy are wound healing and treatment of chronic inflammations. Both conditions are characterised by decreased oxygen tension and acidosis (Karu 1998). In the case of normal regeneration, wound hypoxia is a transient condition which eventually replaced by a normal pO_2 during the final stages of regeneration. The situation is different with chronic injuries. Chronic inflammation is characterised by continued aerobic glycolysis and by a redox shift towards a reduced state. Karu (1998) suggested that when irradiating fresh wounds, the effect of irradiation can be minimal or nonexistent. This happens in cases where the proliferation is active and the regeneration of tissue integrity occurs arbitrating a more or less maximal state.

This may be the reason why there is often no phototherapeutic effect observed when irradiating fresh experimental wounds (Karu 1998).

The results of the present study indicated that no late and early effects in term of protein expression and ALP activities of laser irradiation on human osteosarcoma cell line were found. The present study experienced some limitations. The laser unit was not designed for in vitro use and was broken twice. Due to the small spot size and energy output, only limited numbers of samples could be irradiated in one experiment. Karu (1998) suggested that ambient light should be minimised during the laser experiment and that was not arranged at the beginning. It was difficult to estimate the impact of this parameter.

4.9 Future research

While it is important to continue the studies the cellular response and the interaction between LLLT, attention should also be paid to clinical research. LLLT has been used in many areas including orthodontics. LLLT is a relatively safe therapy. Although the fundamental effects of LLLT on cells remain elusive, a clinical protocol may be developed for the orthodontic situation. Pain reduction during orthodontic treatment, improved bone healing, improved rate of tooth movement, decreased root resorption may only be a few of the clinical situations that could benefit from LLLT. The protocol developed by Kert & Ross (1989) has not been verified by well designed studies. It appears that the choice of dosages and other treatment parameters is based on "clinical judgement and clinical experience". The often mentioned treatment reaction is subjective and not well defined. A well defined and verified guide line for LLLT, possible in treating orthodontic patients is long overdue.

4.10 Conclusions

1. No significant early or late effects of multiple laser irradiations with a GaAlAs continuous wave diode laser, wavelength: 839nmn, output: 90mW, power density: 0.017-0.0249J/mm^2 on protein expression of a human osteosarcoma cell line SAOS-2 were found.

2. No significant early or late effects of multiple laser irradiations with a GaAlAs continuous wave diode laser, wavelength: 839nmn, output: 90mW, power density: 0.017-0.0249J/mm^2 on ALP activity of a human osteosarcoma cell line SAOS-2 were found.

3. There was a trend for laser irradiation at high energy levels to be associated with a decrease in ALP activity one hour after irradiation. However, a general dose-related response could not be identified.

Appendix 1: DMEM (ICN, BioMedical, Australia)

1. Add DMEM — 1 pack
   (powder with L-glutaine without sodium pyruvate and sodium bicarbonate)
2. Penicillin/Streptomycin (50000IU/mL solution) — 10mL
3. Sodium Bicarbonate (7.5% solution) — 20mL
4. Add to 1000mL with Milli-Q water
5. Adjust pH
6. Filter sterilise
7. Supplement with 10% FCS
8. Supplement with 2mL per 100mL with 200mM L-glutamine every 2 weeks

Appendix 2: L-Glutamine (200mM solution)

1. L-Glutamine — 2.922g
2. Add to DMEM solution — 100mL
3. Store frozen for use

Appendix 3: MTT (5mg/mL)

1. MTT — 25mg
   (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
2. PBS — 5mL
3. Vortex
4. Stand 30 minutes at room temperature
5. Filter sterilise
6. Store at 4°C in foil wrapped bottle

Appendix 4: ALP buffer

1. Prepare stock: Glycine 0.1M in NaCl 0.1N
   (7.507g glycine +5.8444g in a litre)
2. Prepare stock: NaOH 0.1N
3. Glycine 0.1M in NaCl 0.1N — 55.7mL
4. NaOH 0.1N — 44.3mL
5. Adjust pH to 10.5
6. Filter sterilise
7. Store at 4°C

Appendix 5: Reagents for SDS-PAGE gel

Stock solution

A. Acrylamide (30% T, 2.67% C)

87.6g acrylamide (29.2g/100mL)

2.4g N'N'-bis-methylene-acrylamide (0.8g/100mL)

Make to 300mL with deionised water. Filter and stored at 4°C in the dark

B. 1.5M Tris-HCl, pH 8.8

27.23g Tris base (18.15g/100mL)

~80mL deionised water

Adjust to pH 8.8 with 6N HCl. Make to 150mL with deionised water and store at 4°C

C. 0.5M Tris-HCl, pH 6.8

6g Tris base

~60mL deionised water

Adjust to pH 6.8 with 6N HCl. Make to 100mL with deionised water and store at 4°C.

D. 10% sodium dodecyl sulfate (SDS)

Dissolve 10g SDS in 90mL water with gentle stirring and bring to 100mL with ddH_2O

E. Sample buffer (SDS reducing buffer) (Store at room temperature)

Deionised water	3.8mL
0.5M Tris-HCl, pH 6.8	1.0mL
Glycerol	0.8mL
10% (w/v) SDS	1.6mL
2-mercaptoethanol	0.4mL
1% (w/v) bromophenol blue	0.4mL
Total	8.0mL

F. 5X electrode (Running) buffer, pH 8.3

Tris base	9g	(15g/L)
Glycine	43.2g	(72g/L)
SDS	3g	(5g/L)

To 600mL with deionised water, store at 4°C

Dilute 60mL 5X stock with 240mL deionised for one electrophoretic run

Appendix 6: Silver stain for mini-protean gel

1. Wash in Milli-Q water for 5 minutes
2. Rinse with ethanol: acetic acid: water (40:10:50) for 30 minutes
3. Soak with Ethanol: Acetic acid: water (5:5:90) overnight
4. Wash in Milli-Q water for 5 minutes
5. Soak in gluteraldehyde (1%) and sodium acetate (0.5M) for 5 minutes

Gluteraldehyde (50%)	4mL
Sodium acetate	8.2g

   Make up to 200mL with water
6. Wash three times in Milli-Q water for 10 minutes
7. Stain with staining reagent for ~ 15 minutes

Conc. NH_4OH	2mL
0.1N NaOH (0.2g in 50mL)	28mL
20% $AgNO_3$ (1g in 5mL H_2O)	3.5mL

   Add 2mL of conc. NH_4OH to 28mL of NaOH solution. Then add 3.5mL of $AgNO_3$ slowly and swirl constantly to mix solution until sign of precipitation. Make up to 150mL with 116.5mL Milli-Q water.
8. Wash four times in Milli-Q water for 10 minutes
9. Develop with citric acid (0.5% w/v) and formaldehyde (0.2% v/v) until bands are clear. (Do not overdevelop. Gel continues to develop after 5% of acetic acid)

Citric acid	0.1g
Formaldehyde	400µL

   Add 199.6mL Milli-Q water
10. Stop the development with 5% acetic acid

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