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Contents

Key words: Urinary incontinence, pulsed electromagnetic field, pelvic floor muscle training.

Implementation of treatment protocol on patients with urinary incontinence.

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ABSTRACT

It is postulated that 30% of adult women in the world are affected with genuine urinary incontinence. Another statistics in the Kingdom of Saudi Arabia supports the same percentage of the affected Saudi women. Different treatment protocols have been proposed for the management of genuine urinary incontinence, but other than exercises, they lack scientific support and research evidence. Pulsed electromagnetic field (PEMF) has been proposed to have a great therapeutic input in people with urinary incontinence through improving the metabolic rate of the pelvic floor muscles to relieve pain and muscle spasm. The aim of this study was to implement the efficacy of pelvic floor muscle training by combination with pulsed electromagnetic stimulation. Twenty patients (male and female) with stress and/or urge incontinence were participated in the study, which was carried out at Physical Therapy Department, King Faisal Specialist Hospital and Research Center. The number of usage pads, urge, and the ability of closing urethral orifice during coughing, sneezing, laughing as well as walking, were used as measuring parameters. Patients taught how to do exercises at home at different levels, and received sessions of PEMF. Results support the efficacy of the treatment plan of exercises and PEMF on controlling urinary bladder in patients with stress and/or urge incontinence. An average of 16-18 sessions received by the patients, were effective to resolve the symptoms in 100% of the treated cases.

INTRODUCTION

Urinary incontinence is defined by the International Continence Society as a condition in which involuntary loss of urine is a social or hygienic problem and is objectively demonstratable [1]. Urinary incontinence is a particularly embarrassing and distressing condition with significant medical, social and economic implications. According to epidemiological studies, it affects the 30% of the adult women in the world are [1].

Physical therapies to treat genuine stress incontinence may include pelvic floor muscle exercises (PFME) with or without biofeedback, electrical stimulation, and weighted vaginal cones [2]. Pelvic floor exercise is known to be an effective treatment for stress incontinence [3], but randomized control trials evaluating electrical stimulation and vaginal cones have given conflicting and inconclusive results, and many of these studies are flawed because of small sample size [3]. In comparison with the effect of pelvic floor exercises, electrical stimulation, vaginal cones and no treatment in women with stress incontinence, Kari et al. [4] reported the superiority of pelvic floor exercises over electrical stimulation and vaginal cones for treatment of stress incontinence. Adverse effects were reported with use of electrical stimulation and vaginal cones but not with exercises. Patients' tolerance for electrical stimulation and vaginal cones was low.

Pelvic floor muscles exercises (PFME) is a well accepted therapy [5]. In 1948 Kegel [6] reported a cure rate of 84% after training of the pelvic floor muscles for women with various types of incontinence. PFME is also used in the treatment of women with mixed incontinence, and less commonly for urge incontinence. PFME have been used in conjunction with different modalities to increase the outcomes. PFME and biofeedback therapy have been used to treat the symptoms of people with urinary incontinence. However, standards of treatment are still lacking and the magnitude of alleged benefits has yet to be established. The limited number of identified trials together with their methodological weakness, do not allow a reliable assessment of the possible role of pelvic floor muscle exercises and biofeedback therapy in the management of people with urinary incontinence. There is a suggestion that some elements of biofeedback therapy and pelvic floor muscle exercises may have a therapeutic effect, but this is not certain. Larger well-designed trials are needed to enable safe conclusions.

Some studies report a significant improvement in incontinence when cones are applied [7, 8, 9]. Other studies report that PFME and the use of cones give a 40-60% decrease in the frequency of urinary leakage and pad changes [2]. Wilson and Borland [9] showed that once women had learned to identify pelvic floor contractility with cones, the majority

continued with PFME alone essentially on the ground of convenience. For follow-up purposes, Olah et al. [10] found an initial drop out rate of 27% that further increased to 42% after six months. Kondo et al. [11] had an initial drop out rate of only 12% but three years later, none of their patients was still using cones while 36% had taken part in some form of pelvic floor rehabilitation. This means that cones have only a role to play for a short term.

Electrical stimulation has been used to excite pelvic floor muscle fibers, leading to muscle contraction and urethral closure. It has been shown that electrical stimulation can aid in restoring continence by stimulating the pudendal nerves and activating pelvic floor muscles [3, 12]. Electrical stimulation currents can be delivered intravaginally with a vaginal plug, percutaneously with a surface electrode or needle, or intra-anally with an anal electrode. General success rates from 50% to 90% have been reported for electrical stimulation treatment of stress incontinence [13, 14]. Two main problems limit the usefulness of electrical stimulation therapy. First, the insertion of electric probes is often uncomfortable for the patient. Second, electric current induced by electrical stimulation weaken due to the impedance of the tissues between the electrodes and the target nerves. This decrease in magnitude is marked in bone-encased nerves, such as the nerves of the spinal cord or of the motor cortex of the brain, but is also significant when the current must pass through skin and subcutaneous tissues, both of which have high impedance values. To counteract the decrease in electrical magnitude, strong electrical currents must be used during therapy, activating pain receptors and leading to discomfort.

Unlike electric fields, magnetic fields penetrate all body tissues without alteration, decreasing in magnitude proportionally to the inverse square of the distance between the target tissue and the magnetic source. Magnetic stimulation, therefore, promises distinct advantages over electrical stimulation to induce muscle contraction is affected by the magnetic field will be the subject for future studies.. Assuming charged particles are present, a changed magnetic field will lead to a flow of electrons, inducing current in human tissues. Subsequent depolarization of motor nerves triggers release of neurotransmitters, leading to muscle contraction [15]. While the overall mechanism by which magnetic stimulation causes muscle contraction is similar to the mechanism by which electrical stimulation achieves the same goal, magnetic nerve stimulation is noninvasive and painless. Since magnetic fields penetrate clothing and are undeterred by impeding tissue, they can penetrate into deep nerves without alteration of field strength [16]. This paper reports the results of a clinical study in which the effectiveness of magnetotherapy in incontinence has been assaied.

MATERIALS AND METHODS

Patient enrollment

Twenty patients, males and females, age range 43.6 ±14.09 years, participated in this study. Patients were diagnosed and referred from department of urology. Patients were informed about participation in the study and signed a consent form. Selection criteria: Patients with symptoms or uro-dynamic diagnoses of stress, urge or mixed incontinence were included in the study.

Research Design and Instruments and Protocols.

This study was conducted at the King Faisal Specialist Hospital & Research Center. The clinic operated on an appointment system with appointments on the same day. It was a pre-test, post-test research design.

First visit and treatment program:

- Patients were diagnosed by urologist and/or gynecologist, and then referred to Physical Therapy Department for treatment.
- Patients were screened by the therapist in order to meet the requirement of the sample.
- Patients were informed about the study and the treatment program.

Day	Date
6-8 am	
8-10 am	
10-12 am	
12-2:00 pm	
4-6 pm	
6-8 pm	
8-10 pm	
10-12 pm	
Over night	
Pads used	
Comments	

Weekly Bladder Diary
The following abbreviations were used in the second column as a reference.
L= leaking during ADL (coughing, sneezing, laughing, walking, urge)
P= number of used Pad

- Consent form was signed by the patients.
- Patients were interviewed for questioning and determining the base-line of the current condition according to the criteria of the study.
- A weekly diary to be filled-in was given to each patient. Information from the patients was collected weekly.
- The treatment consisted in exposure to PEMF for 16-18 of 25 minutes each. The source of PEMF was an ASA Co. Magnetic Therapy apparatus (ASA Srl, Italy) (Fig. 1) and pulsed electromagnetic (PEMF). The PEMF was set up at frequency 100Hz, intensity 75% for 25 minutes. Moreover, a PFME program based on Kegel program [6] was practiced and given to all the patients. They performed it on regular basis (3 times/day).

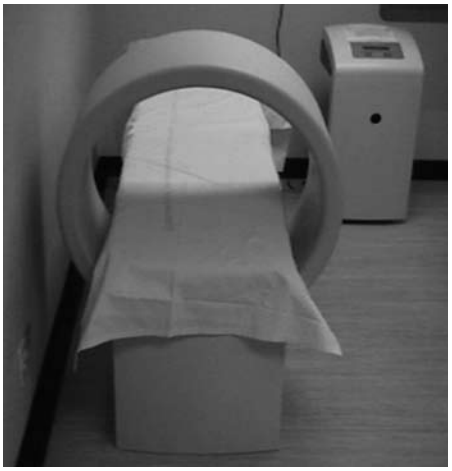

Fig. 1. ASA Magnetotherapy

EVALUATION PARAMETERS

Urethral pressure profilometry was assessed with a symptomatically full bladder at rest in the sitting position, at maximal pelvic floor muscle contraction (the patient was asked to "hold urine"), during repeated coughing, and during coughing while contracting the pelvic floor muscles in an attempt to hold urine [17]. Patient self-report for these parameters were taken for assessment in pre- and -post treatment. Number of used pads, urge and urethral orifice control during cough, sneeze, laugh and walking (before and after treatment) were used as measuring parameters.

Data analysis:

The statistical analysis was done by using the statistical software SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

Table 1 shows the overall averages, standard deviations, minimum and maximum values of all the variables under study for all the patients before and after treatment. It is clear that all the averages have dropped after treatment for all the variables. Then paired t-test was done for all the variables to investigate the significant difference after treatment. Table 2 shows the results of the paired t-test and we can see that the following variables: cough, laughing, urge and pad were all significantly different after treatment ($P < 0.05$).

RESULTS

All twenty patients (male and female) completed the study. None of the subjects was found to have intrinsic sphincter deficiency, defined as maximum urethral closure pressure 20 cm of water. The study was designed on the basis of 3x1 variables. The dependent variables were number of used pads, urge and urethral orifice control during coughing, sneezing, laughing, and walking. The dependent variables were measured before and after treatment (Tables I and II). The independent variable was the treatment intervention, which included PEMF and PFME.

The comparison between the means of the considered parameters before and after the treatment showed a very significant difference (553-to-38¾). See Fig. 2, Tables I and II.

T-test was used for performing statistical analysis of the differences in the evaluation parameters before and after treatment. The findings demonstrated that there was

Variable	*Pre Mean±STD*	*Post Mean±STD*	*Minimum Value*	*Maximum Value*
Cough	3.4±5.5	0.1±0.5	0	14
Sneezing	2.0±4.3	0.0±0.1	0	14
Laughing	3.0±5.7	0.2±0.6	0	14
Walking	2.7±6.0	0.0±0.1	0	21
Urge	4.0±5.6	0.1±0.3	0	21
Pad	12.6±17.1	1.8±5.5	0	49

Table 1. Pre and Post Averages for All 20 Patients.

Variable	*Differences Mean±STD*	*t Value*	*P-value*
Cough	3.3±5.6	2.62	0.0168*
Sneezing	1.9±4.3	2.06	0.0530
Laughing	2.7±5.8	2.10	0.0495*
Walking	2.7±6.0	1.99	0.0615
Urge	3.9±5.6	3.09	0.0061*
Pad	10.8±14.6	3.30	0.0038*

Table 2. Paired t-test Results.
*Statistically different

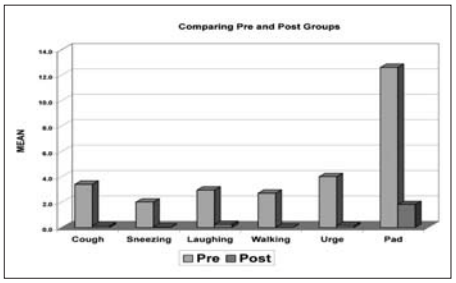

Fig. 2. Evaluation parameters before and after the treatment.

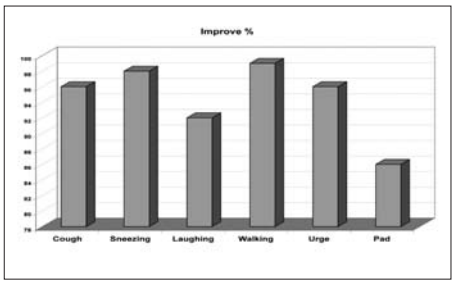

Fig. 3. The percentage of improvement in the evaluation parameters after the treatment.

parameters examined (Table III), therefore the therapy was effective in reducing the symptoms. All dependent variables showed very significant differences before and after treatment. The used pads were dramatically decreased after treatment; indicate the efficacy of treatment tools on solving patient's problem, to become dry. The high percentages of improvement indicate great changes of patient's lifestyle after treatment intervention. The changes of stooping activities (cough, sneezing, laughing), physical activities (walking), and number of used pads indicate a quality of life for the patient with incontinence.

DISCUSSION

The patient with uncontrolled bladder may complain of frequent episode of urination during certain activities of daily living. These activities may require straining and increase of intra-abdominal pressure like coughing, sneezing, laughing, walking and/or running. Different treatment methods have been used to solve the problem as pelvic floor muscle exercises, electrical stimulation and magnetic stimulation for pelvic floor muscles, vaginal weight cones, and behavioral

therapy as biofeedback technique, meditation and relaxation techniques. Other than exercises, no evidence based behind any of these therapeutic way, has been strongly supported.

In this clinical study, the urodynamic parameters measured before and after the proposed therapeutic protocol (PEMF + PFME) were found to be very different.. As shown by the results, urodynamic symptoms of the patients subjected to the treatment protocol significantly decreased (p value for cough ≤0.0162, sneezing ≤0.0466, laughing ≤0.0471, and walking ≤0.0540). All patients became dry and they were able to go back to a life style similar to the one they practiced before the disease occurrence (p value for pad usage 0.0131). Patients who suffered from urge, obtained excellent results (p value for urge ≤0.0058). Our data suggested that the proposed treatment protocol (PEMF and PFME) was very effective in solving the problems that face the patients with urinary incontinence.

Magnetic stimulation has great potentiality in treating stress urinary incontinence [16]. The physiological background of the PEMF effect may be based on the fact that it is thought to enhance the gas exchanges at membrane level, and thence the metabolic rate of the tissues. This technique, when applied to the pelvic area, was found to increase the maximum intraurethral pressure during stimulation as well as the maximum urethral closure pressure after treatment in healthy, young volunteers [16].

On the other hand, a research done by Jonathan et al. [18] demonstrated that, even if the magnetic shoe cushion device does not stimulate a pelvic floor contraction, it appears to increase the efficacy of knack maneuver. What the shoe cushion device achieves is an enhancement of the contraction of the pelvic floor muscles during coughing. This provides adequate support of the urethra when it is most needed. The long-term effects as well as the precise electrophysiological mechanism by which muscle contraction is affected by the magnetic field will be the subject for future studies.

More than fifty years ago, Kegal [6] suggested exercises to strength pelvic floor muscles. Pelvic floor muscle training resulted better than no treatment or placebo treatments. The limitations of the evidence available means that it is difficult to judge if pelvic floor muscle training was better or worse than other treatments. Most trials to date have studied the effect of the treatment in younger, premenopausal women. Side effects of pelvic floor muscle training were uncommon and reversible. A number of the formal comparisons should be considered with caution due to statistical heterogeneity, lack of statistical independence, and the possibility of spurious confidence intervals in some instances. Pelvic floor muscle training appeared to be an effective treatment for adult women with stress or mixed incontinence. The role of pelvic floor muscle training for women with urge incontinence alone remains unclear. Many of the trials were too small, with poor reporting of allocation concealment and masking of outcome assessors. In addition there was a lack of consistency in the choice and reporting of outcome measures, that made data difficult to combine. Methodological problems limit the confidence that can be placed in the findings of the review. Further, large, high quality trials are necessary.

The physiologic basis of the efficacy of exercises on pelvic floor muscle is that fast twitch muscle fibers contract during coughing, while slow-twitch muscle fibers sustain contraction around the urethra and add basic muscle tone to the pelvic floor. Performance of pelvic muscle exercises strengthens the levator-ani muscles by repetitive voluntary contractions. These exercises, strength slow-twitch muscle fibers only, unless the patient is instructed to alternate slow tonic contractions with the fast ones. The efficacy of exercises and PEMF separately on urinary incontinence is beyond the scope of this research, but we demonstrated that the cumulative effect of both of them may reach to the positive results. In conclusion, the therapeutic effect of exercises and PEMF in management of genuine stress incontinence may open a new avenue for safe, noninvasive and painless management. Larger well-designed trials are needed to enable safe conclusions.

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Key words: Laser therapy, muscle atrophy

Does laser prevent physiologic muscle atrophy? An histological study.

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ABSTRACT

Physical therapists usually confront with the dilemma of physiologic muscle atrophy, especially after limb immobilization. Exercises as well as electric stimulation are the preferred treatment routine to prevent disuse muscle atrophy. In the histological study here presented, twenty albino rats, equally divided into two groups: (1) control group, and (2) laser group were used. In all the albino rats the whole right leg was immobilized in a plaster cast, started above the foot and ended below the hip joint. For the experimental group, a window was opened on the front of the thigh over the quadriceps muscle for treatment intervention. The experimental group received daily LASER treatment for one month. At the end of the study, animals were sacrificed and a histological study was done to measure the dependent variable of the study, which is the size of the muscle fiber. The aim of the study was to determine the effect of laser therapy on prevention of muscle atrophy in simple physiological immobilization versus control group. Results of this study revealed that immobilization for one month produced a relative muscle fiber size variation (atrophy) of 30.7% in the immobilized limb, and 20.4% in the immobilized limb exposed to the laser treatment. In conclusion, laser therapy may have a significant effect in prevention of muscle atrophy in physiological immobilization.

INTRODUCTION

The role of Physical Therapy during the period of immobilization is to prevent or decrease the deleterious effects of immobility and disuse on bone, joint and soft tissue structures. Muscle atrophy is one of the most common problems seen at the end of immobilization period. The longer the immobilization period is, the more the adverse effects on soft tissues are. The main goal of treatment during the immobilization period is to keep the skeletal muscles and their blood supply, like pumping effect, in or around its normal level [1]. Physical therapists usually apply active exercises and/or electrical stimulation for preventing of muscle atrophy for the immobilized muscles. Laser is a relatively new therapeutic tool. It has been used to reduce pain and to accelerate healing process of wounds. In a Russian research study, Nemtsev reported that HeNe laser may be recommended for atrophy prophylaxis and treatment in cases of hypokinesia and weightlessness induced atrophy [2]. Whilst the Food and Drug Administration (FDA) in the USA has still to approve laser therapy, the modality has found increasing application by physical therapists, dentists, acupuncturists, and some physicians, for a range of conditions including the treatment of open wounds, soft tissue injuries, arthritic conditions and pain associated with various aetiologies [3]. The potential of relatively low intensity laser irradiation applied directly to tissue in modulating certain biological processes, in particular to photobiostimulate the wound healing process, has been mentioned [4].
The aim of the study here presented was to determine the effect of laser therapy on prevention of muscle atrophy in physiological immobilization.

MATERIALS AND METHODS

DESIGN OF THE STUDY. It was 2x1 pre-test/post-test research design. Laser therapy and sham treatment "control group" were the independent variables, and the muscle fiber size was the dependent variable.
EXPERIMENTAL MODEL. The experiment was carried out on 20 male albino rats. Their body weight ranged between 120-150 grams at the beginning of the study. The rats were divided into two groups:
Group I "Control group". Ten rats were immobilized by a plaster cast covering the whole right lower leg, started above the foot and ended below the hip joint, keeping the ankle joint & the hip joint free from immobilization. The period of immobilization continued for one month without any treatment.
Group II "LASER therapy". Ten rats were immobilized (same as in group I), but a window was opened on the front of the thigh over the quadriceps muscle to apply daily laser therapy for ten minutes during the period of immobilization (one month).
INSTRUMENT. Laser therapy unit LTU-904 is a portable, simple-to-use unit. Activated by a finger-tip control, the hand held Laserex LTU- 904 consists of a pulsed infra-red laser whose radiation penetrates into the affected tissues to a depth of 20-

30 mm. The advantages of a pulsed Ga-As infra-red laser (λ=904 nm), as compared to a continuous He-Ne laser (λ=632 nm) are in the greater depth of penetration and selective biological absorption. Specifications for LTU-904 are:

- Laser type: Gallium arsenide (Ga-As) Laser diode
- Laser wavelength: 904 nm
- Peak power: 5 W
- Mean Output power: 5.0 mW
- Pulse repetition rate: 5000 Hz
- Pulse duration: 200 ns
- Warning signals: Inbuilt emission detector and visual/audible warning signal.
- Mean fluence: 20 mW/cm2

PROTOCOL.

All the rats were kept in the same conditions, except laser treatment. The rats in the experimental group received daily treatment of laser therapy for ten minutes through the window in the plaster cast, which was opposit to the motor point of the quadriceps muscle. The rat in the control group received a sham treatment. At the end of the experiment, all the animals were sacrificed with an overdose of ether and the quadriceps muscles were dissected out for histological examination. Jaffe [5] method was used for tissue preparation. Histological examination were subjected to the morphometric study "the qualitative description of a structure" [6, 7]. Measurement of the muscle fiber diameter was made by means of a micrometer disc that was placed in the ocular disc of the microscope. The disc is usually calibrated as a line divided into 100 units. This calibration is done for standardization of the measurements taken by the ocular micrometer[8]. All the results were tabulated and the mean number of the maximum muscle fiber diameters was calculated.

RESULTS

The difference in size of muscle fibers between immobilized and non-immobilized limb in control and laser-therapy groups was calculated to detect the percentage of muscular atrophy in the right immobilized limb in relation to the left non-immobilized one. The change in muscle fiber size in each group was compared to determine the effect of laser-therapy on prevention of muscle atrophy in physiological immobilization.

GROUP I (CONTROL).

The data reported in Table I show that seven animals survived and were investigated. The maximum muscle fiber diameter (MMFD) in the right immobilized limb ranged between 11.8 and 18.1μ with a mean value of 14.6 with SE=0.2. The MMFD in the left non-immobilized limb ranged between 16.7 and 26.7μ with a mean value of 21.3μ with SE=0.3. The difference in MMFD between the right and left side ranged between 4.8 and 8.7 with a mean value of 6.6 with SE=0.2. The percentage of atrophy in this group ranged between 15.4 and 42.3% with a mean value of 30.7% with SE=0.5. The decrease in muscle fiber size due to muscular atrophy in the right side was statistically significant as t=19.5 and p<0.00001 (Table III). The microscopic examination of a Transverse Section (TS) by a photomicrograph showed a great decrease in muscle fiber size in the right immobilized limb compared with the left non-immobilized side in the control group.

GROUP II (TREATED).

Seven animals survived and were investigated. The difference in MMFD between the right and left side ranged between 2.0 and 11.2μ, with a mean value of 5.3 with SE=0.2 (Table II). The decrease in muscle fiber size, due to muscular atrophy in the right side, was statistically significant as t=9.5 and P<0.00001 (Table III).

The decrease in muscle fiber size in the right immobilized limb, when compared with the left non-immobilized side, was analyzed by photomicrograph of a TS with the same magnification for each side.

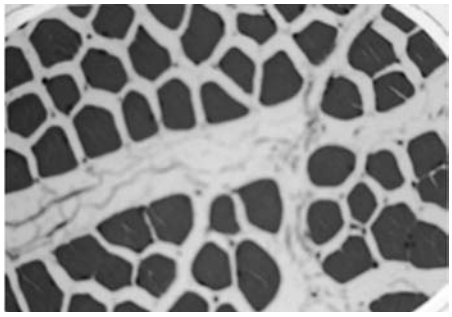

Fig.1. A photograph of a TS of the normal muscle fiber size (left side) from a male albino rat showing normal muscle fibers with peripheral nuclei. The connective tissue endomysium & perimysium are normal (H&Ex200)

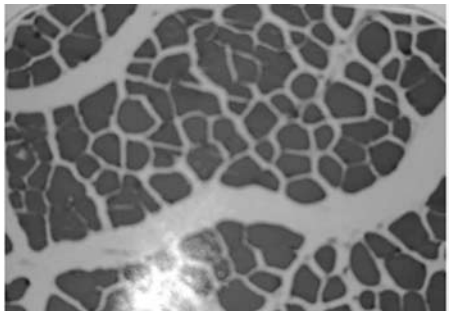

Fig.2. A photograph of a TS of the immobilized muscle fiber size (right side) from a male albino rat showing atrophic muscle fibers with peripheral nuclei (H&Ex200)

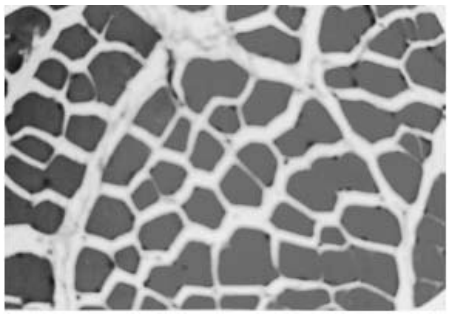

Fig.3. A photomicrograph of a TS of the normal muscle (left side) from a male albino rat showing normal muscle fiber size with peripheral nuclei. The connective tissue endomysium & perimysium are normal (H&Ex200)

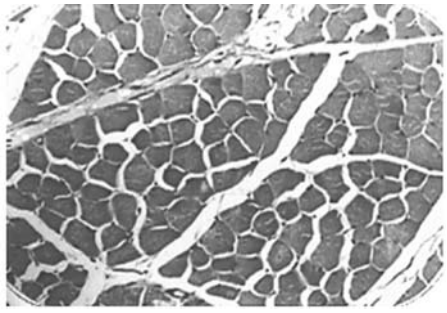

Fig.4. A photomicrograph of a TS of the immobilized muscle fibers (right side) from a male albino rat showing the difference in muscle fiber size after laser- treatment (H&Ex200)

DISCUSSION

Limb immobilization is commonly used to protect fractured bones and injured tissue. The most common complication of immobilization is the muscle wasting that occurs due to a decreased muscle use. The results of the current study supports the deleterious effects of disuse and immobilization on the muscle fiber size, which lead to atrophy of the immobilized muscles. In fact, in both the groups studied there was a significant decrease in the MMFD of the immobilized limb in comparison with the non-immobilized one.

As regards the effect of laser-therapy on muscle fiber size, our findings might support what was reported first by Nemtsev [2], who recommended laser-therapy for atrophy prophylaxis and treatment in cases of hypokinesia and weightlessness induced atrophy. In fact, even if the reduction in muscle atrophy by lasertherapy cannot be considered significant from the statistical point of view ($p \geq 0.05$), however we found that the decrease in the diameter of muscle fibers in the immobilized leg of treated animals was about 20% versus the 30% monitored in control animals. The positive effect the laser treatment seemed to have on trophism of muscle fibers is in agreement with data shown by other authors.

The biophysical effects of lasertherapy on muscle have been widely reported in literature [9, 10]. Recently, the efficacy of 780-nm lasertherapy on peripheral nerve regeneration has been demonstrated [11]. Moreover, low level laser therapy seems also to affect growth and regeneration of capillaries [12].

Many studies have been carried out on the effects of laser on cell growth. The data showed that the growth stimulation was accomplished by an increase in the respiration activity and by synthesis process in the cell, while accumulation of the toxic intermediate of oxygen metabolism and degenerative processes decreased. [13]. Recent research confirmed that laser irradiation can enhance proliferation and metabolic processes in cells [14, 15].

Table I. Maximum Fiber diameter of the quadriceps in control group.

Animal #	Right	Left	Differ.	%
1	15	22.4	7.4	33.1
2	13.7	18.7	5.0	26.7
3	18.1	26.7	8.7	32.4
4	16.5	19.5	3.0	15.4
5	14	24.2	10.2	42.3
6	13.2	20.7	7.8	36.2
7	11.8	16.7	4.8	29.0
Mean	14.6	21.3	6.7	30.7
SD	2.1	3.4	2.5	8.4
SE	0.2	0.3	0.2	0.5

The difference in MMFD between the right and left side ranged between 15.4%-42.3% with a mean value of 30.7 with SE=0.51. The decrease in muscle fiber size due to muscular atrophy in the right side was statistically significant as t=19.5 and P<0.00001.

Table II. Maximum Muscle Fiber Diameter (MMFD) of the quadriceps in the laser-treated group.

Animal #	Right	Left	Difference	%
1	22.3	25.9	3.6	13.8
2	16.8	23.6	6.7	28.6
3	20.7	22.7	2.0	8.8
4	21.0	25.3	4.3	17.1
5	19.3	24.5	5.1	21.1
6	22.5	33.7	11.2	33.1
7	16.6	20.8	4.2	20.4
Mean	19.9	25.2	5.3	20.4
SD	2.4	4.1	3.0	8.4
SE	0.3	0.4	0.2	0.7

The values show the difference in MMFD between the right immobilized limb and the left non-immobilized limb in the laser-treated group.

Table III. Mean values of MMFD in right and left side in the two groups.

	Control Group		LASER Group	
Side	Right	Left	Right	Left
Mean	14.6	21.3	19.9	25.2
SD	2.1	3.4	2.4	4.1
SE	0.2	0.3	0.3	0.4
t	19.5		9.5	
P	<0.0001	<0.0001	<0.0001	<0.0001
Sig.	*	*	*	*

It is showing the significant different. The results indicated that there was a significant difference in size between the right and left sides in all groups.

In conclusion, this experimental study provides the clinicians with a scientific piece of information about the application of laser therapy for preventing muscle atrophy. Prolonged immobilization for one-month lead to 30% muscle atrophy of its original size, but the daily exposure to laser treatment reduce muscle atrophy to 20%. Further studies are in progress, with an increased number of cases, in order to improve the statistical significance of the results.

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Key words: Low Level Laser Therapy, low power laser, low level laser

The benefit of using Low Power Laser in the treatment of various illnesses – an overview.

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ABSTRACT
The aim of the article is to give an overview of the interesting field of Low Level Laser Therapy. Applications like the treatment of inflammatory processes, acute myocardial infarction, acute ischemic stroke and others are possible but still rarely used in clinical routine. This paper summarizes the most important applications and mechanisms underlying the effects of low level laser.

OVERVIEW
Biomodulation, i.e. the use of Low Level Laser Therapy (LLLT) or light sources of a certain wave length, is a well known method to treat inflammatory processes [1-4], to accelerate wound healing and to alleviate pain in various conditions such as folliculitis, abscess, or rheumatoid arthritis (see Table I). Studies have suggested that infrared laser therapy could also be beneficial for the treatment of acute myocardial infarction, acute ischaemic stroke, injured peripheral nerves and spinal cord injury [5, 6]. The method has become relatively popular in recent years, especially in Europe.
A large number of studies, clinical evaluations and more than 100 double-blind studies have demonstrated the effective use of laser therapy in pathologies mentioned above, especially in the fields of dermatology, neurology, surgery, rheumatology, traumatology, gynaecology, dentistry and veterinary medicine.
The method has been successfully used in more than 40% of the rehabilitation centres in Great Britain and in approximately 30% of the dentistry clinics in Scandinavia for many years [4]. A Norwegian master thesis from 1997 [7] formed the basis for the treatment approval by the Norwegian health insurances.

In spite of many applications in humans the biomodulative effect of low level laser therapy has still not been completely understood. The spectrum of visible to infrared light can cause stimulation as well as inhibition of various organisms [1, 8, 9]. Photobiomodulation involves increased adenosine triphosphate (ATP) formation after energy absorption inside the mitochondria [10, 11]. A compound that absorbs energy in the spectral region of interest is known as a chromophore. There is evidence that suggests that the primary mitochondrial chromophore for photobiomodulation is cytochrom c oxidase [10, 11]. Endogenous porphyrins and cytochromes are probably photoabsorbers. Components of the respiratory chain like flavines and cytochromes might be the first step of a beginning photo-induced reaction [1, 8-11]. In vitro experiments showed that LLLT biomodulation can stimulate the emission of growth factors [12, 13], as well as cell proliferation [8, 9, 13] and collagen synthesis [14].

One group found out that low-level laser irradiation can enhance the proliferation of mesenchymal and cardiac stem cells, which may have an important impact on regenerative medicine [6].
Other studies demonstrated that LLLT improves the local blood microcirculation [15-17]. This phenomenon might be the explanation for improved wound healing and local pain control by the use of LLLT.
As mentioned before, an increased ATP production after laser application might explain beneficial effects of LLLT after stroke [10, 15]. The NEST-1 study, a multicenter prospective double blind randomized trial, involved 120 patients with ischaemic stroke. The randomisation ratio was 2:1, with 79 patients in the active treatment group and 41 in the sham (placebo) control group, 70% of patients from the active group had a successful outcome in comparison to 51% in the control group. The study concluded that infrared laser therapy is safe and effective for the treatment of ischaemic stroke in humans when initiated within 24 h of stroke onset [15].
A significant long-term functional neurological benefit following traumatic brain injury was found in mice when treated with low level laser 4 h after the trauma [6].
In vivo studies have also suggested that LLLT could be beneficial for the treatment of acute myocardial infarction [18].

The effects of LLLT biomodulation or of other light sources like LED [13] are

Effect	Mechanism	Examples
Inhibition of inflammatory reaction	• Improved phagocytosis • Inhibition of mast cell degranulation • Activation of immune cells via increased mobilisation of leucocytes • Increased microcirculation via vessel dilatation • Decrease of inflammatory swelling via stimulation of the lymphatic flow • Reduced prostaglandine synthesis	• Folliculitis, abscess, boil, carbuncle • Viral dermatoses (warts, herpes simplex, zoster and genitalis) • Rheumatoid arthritis I+II, arthritis septica and allergica • Tendinopathies, achillodynia, coracoiditis etc. • Adductor muscle syndrom • Tonsillitis, otitis, rhinitis etc. • Mucositis after radiotherapy and chemotherapy
Analgesia, hypalgesia, pain inhibition	• Improved beta-endorphin distribution • Increased ATP-production (cell energy) • Increased measurable potential at nerve cell membranes • Muscle relaxation and increase of the stimulus threshold of nerve cells for pressure pain • Decrease of pain	• Peripheral polyneuropathy • Carpal tunnel syndrome, tarsal tunnel syndrome • Muscular tenseness • Myofascial pain syndrome, fibromyalgia • Cervical and lumbar syndrome • Facial neuralgia (trigeminus neuralgia) • Facial palsy
	mediators (e.g. substance P) • Reduction of trigger- and tender-point activity • Activation of acupuncture points	• Intercostal and zoster neuralgia • Traumatic and postoperative pain • Needle substitute
Tissue regeneration	• Enhanced mitosis rate and collagen synthesis, activation of fibroblasts, chondrocytes, osteocytes etc. • Enhanced ATP-production • Improved granulation and epithelisation • Improved peripheral nerve regeneration after trauma • Reduced degenerative CNS-processes • Supported survival of brain cells after transient ischaemia • Reduced or eliminated scarring	• Improved wound healing after injury or postoperative • Decubitus, burns, rhagades • Ulcus cruris and diabetic ulcer • Muscle fibre and ligament rupture, cartilage lesion • Chondropathy, arthrosis • Fracture, disturbed osteosynthesis • Infarct rehabilitation
Circulation improvement	• Improved lymphatic drain • Enhanced microcirculation • accelerated resorbtion of haematomas • Decreased release of vasoactive amines • Increased hyaluronidase activity	• Postthrombotic lymphatic edema • Dizziness, tinnitus, migraine • Chronic postmastectomy lymphatic edema • Posttraumatic swelling

Table I. Indications for laser therapy as cited in the international literature. Laser therapy is a regulatory medical treatment modality which is applied in most of the medical disciplines: dermatology, traumatology, sports medicine, orthopaedy, dental medicine, urology, gynaecology, general medicine, veterinary medicine, physiotherapy, nature medicine etc.
(Adapted from the Lasotronic web site)

often used in vivo in Europe, especially in the treatment of herpes zoster, diabetic ulcers, burns, wound healing disorders, pain and inflammatory processes [13, 16, 19-22].

Despite its longterm use, there still is an on-going controversy in scientific medicine regarding the application of laser biomodulation therapies with low power laser light or other monochromatic light.

It is important to emphasize that light therapy with different wave lengths has been accepted as a low-risk treatment by the FDA [13] and that the application of light as a therapy method has been approved [13].

Low Power Laser Therapy evokes a clinical effect without thermic side effects [16] and at relatively low costs (see Table II).

Acute pain due to polyarthritis, myogelosis, tennis elbow, capsule distensions and tendosynovitis is conventionally treated with cortisone and analgetics. This conventional therapy is, however, frequently ineffective and may moreover cause severe side effects, not to mention the considerable cost factor. The possibility to treat these symptoms fast, easily and cost effectively with low power laser offers an effective therapeutic alternative. Another advantage is that the therapy can be repeated as there are no known side effects.

Mastitis caused by radiotherapy after breast conserving surgery occurs in 20% of patients [23]. Its therapy with cortisone and antibiotics is also costly and may lead to considerable side effects. The treatment of radiotherapy-induced mastitis with LLLT improves the quality of

Explanation of costs		Laser size	
	small	medium	large
Acquisition value - average in Euro	2000,00	3000,00	13000,00
Economic life time - in years	5	5	5
Leasing duration - average in years	2	3	4
Monthly costs based on the life-time - without interests in Euro	33,33	66,67	216,67
Monthly costs based on the life-time - with 5% interests in Euro	41,67	83,33	270,83
Treatments per month	100	100	100
Amortization costs per treatment - in Euro	0,42	0,83	2,71
Average treatment time - in minutes	15	15	20
Time costs (120 Euro/hour)	30,00	30,00	40,00
Total costs per therapy - in Euro	30,42	30,83	42,71
Small: handheld laser devices for acupuncture, 30-70mW			
Medium: table stations with handheld sensor, 50-300mW			
Large: Scanner devices with automatic, up to 500mW or 4W			

Table II. Cost evaluation of laser therapy (By courtesy of Felix Kramer from Lasotronic)

life and is very economical [16].

Other side effects of radiation therapy, especially in patients with tumours of the head and neck region have also been successfully treated with LLLT at low costs [24].

To summarize, biomodulation by LLLT has been demonstrated to be an effective treatment option for inflammatory processes and various other illnesses.

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Key words: Intensity modulation of light, Laser-induced heating, laser-tissue interaction, CO_2 laser.

Effect of intensity modulation of a CO_2 laser beam on the temperature of a targeted thermal sensor simulating biological tissue.

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ABSTRACT

The conditions and characteristics of thermal / temperature response of a bulk PTC-type thermal sensor head (thermistor) irradiated by a CO_2 laser beam are presented. Unmodulated (CW) and square-wave intensity modulated beam are compared. The results are evaluated by considering the sensor head as a physical mass and as simulating material for biological tissue of medical interest. It is thus derived that the intensity modulation, despite a slight lowering of the achievable temperature level, offers the advantages of improved temperature-to-optical power linearity, suppression of the corresponding saturation effects, and capability for temperature modulation.

INTRODUCTION

It Is well known that numerous interactions of a laser beam with biological tissue, for medical treatment, are mainly based on beam-induced thermal processes. Accordingly, the corresponding temperature ϑ and its rise created in the irradiated region and/or its adjacent layers play an important role for the achievement of the desired result (1-7). Indeed, the mean steady-state value of the tissue temperature, particularly when attained through absorption processes, determines the kind of the finally occuring effect which, for instance, may be coagulation or hemostasis if 60° C < ϑ < 100° C, cell water evaporation at 100°C, and pyrolysis or carbonization of the tissue at even higher temperatures (1-4. 8).

The above tissue temperature and the extent of its corresponding thermal effects are strongly influenced by the interaction time and the temporal form of the laser pulses directed to the tissue (1-3, 9). This fact implies that the timing of tissue temperature and its variations provoked by a repetitive/pulsed or a modulated laser beam may have significant influence on the effects in question. Unfortunately, as yet, in the field of clinical applications, modeling studies, of beam-tissue interaction for determining optimal treatment modalities exist only for the simple cases of unmodulated continuous-wave (CW) or one-pulse laser beams with the real tissue roughly simulated by a single plastic material such as, e.g., swollen gelatin, ester methacrylates, or other (4, 6, 7, 10). By contrast, in the cases of laser beams with more complicated timing, such as the multiple-pulses or intensity-modulated (IM) beams, no reliable modeling is in effect possible and therefore no trustworthy prediction of the corresponding tissue temperature can be made. This difficulty is enhanced by considering the eterogeneity of almost all living tissues (1-3).

In consequence, the implantation in vivo of an appropriate thermal sensor head (TSH) near to the irradiated tissue and the corresponding continuous monitoring of the actual temperature frequently constitutes the most reliable method for supervising laser-tissue interactions in the presence of thermal effects, particularly with variable/modulated laser light. It is clear that, under these conditions, the thermal behaviour of the TSH mass itself (characterized by parameters such as its heat capacity, time of response, etc) play an important role in the correct evaluation and interpretation of thermometric results during the laser treatment. Moreover, in several types of thermal sensors, the thermally sensible medium is given the form of a relatively bulky head or it is encapsulated in such a head made by plastic material of dark colour, which is

often the case of customary semiconductive thermistors (11-13). In such cases, the material of the thermal head can possibly simulate successfully enough a biological tissue and therefore the experimental study of temperature variations of such a head submitted to direct irradiation by a laser beam could provide a reliable prediction of the features of thermal interaction between this beam and the simulated tissue. This investigation would be even more useful in the case of modulated laser light, for which modeling data are absent or unreliable.

A first attempt in this direction has been made recently by Theofanous (14) using an electro-optically modulated Krypton laser beam incident on a thermistor head. However, in this trial a complicated set-up of external electro-optic modulation with sinusoidal format, which is rather unusual, was employed and moreover the spectral output of the Kr laser (red lines) did not match well the spectral absorption of the thermal head, which was composed of black-coloured material. Therefore, we have undertaken a similar study using a CO_2 laser beam attacking a bulk-type PTG thermistor head, the black bakelite—like material of which can simulate acceptably dark biological tissues in thermal interaction with a CO_2 laser beam.

In this paper, after a brief consideration on the features of heat deposition in tissues under modulated laser beam, we present the conditions and results of the above experimental investigation with the CO_2 laser employed under stepwise CW regime and under square-wave intensity modulation. Results on the transient and steady-state temperature response are given for various modulation frequencies.

Laser-induced temperature and timing control.

The value of temperature induced by a laser beam on an irradiated mass (thermal sensor head or simulated biological tissue) depends on the transfer of energy, which follows the general scheme depicted in Figure 1, with the final interaction effect resulting from the combined action of reflection, absorption, and scattering of the laser light (4). Among these phenomena that of absorption is the principally responsible for the elevation of temperature.

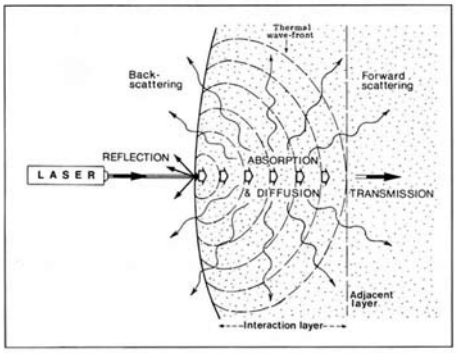

Fig. 1 - *Typical scheme of thermal interaction between a laser beam and a sensible material (thermal sensor mass or biological tissue). The material in question is assumed homogeneous, so as the thermal wave-front propagates uniformly throughout the material.*

Generally speaking, the temperature of the irradiated mass is determined or affected either by material factors, which are related to the target material, or by beam-related factors, characteristics of the laser beam itself. Among the material factors, the more important are the absorption coefficient, the surface temperature, and the thermal conductivity of the material under irradiation (15). Note that thermal conductivity determines the amount of heat transmitted (undesirably) to the adjacent layers of an attacked tissue and therefore plays a significant role in surgical operations (16). On the other hand, among the beam-related factors, the more critical one is the timing control of the beam and it has been clearly ascertained that the steady-state temperature of an irradiated tissue decidedly depends on the ratio of the laser-tissue interaction time to the thermal diffusion time τ_a of the attacked tissular material (5-9, 17,18).

Unfortunately, the theoretical study of the above influence of timing control, has not but small practical use as no reliable information on absorption or scattering parameters of tissues is currently available and the corresponding transport equations require elaborate mathematical procedures (19). Therefore, as yet, investigations of this kind have been performed mainly in the experimental field and moreover – for simplicity – only with interrupted CW and spontaneously / autonomously pulsed or Q-switched lasers.

By contrast, to the authors' knowledge, investigations using intensity modulation (IM) of the laser light, for external control and timing of its optical power or intensity, are lacking in the literature. This light modulation offers a number of advantages (capability of freely adjusting the period and height of laser pulses, possibility of commanding the beam, etc) and therefore has been considered in the present work.

Experimental system and procedure.

The experimental set-up used in the present work is depicted schematically in Figure 2. The beam of a CO_2 laser source, after passing through a series of Si attenuation filters (which control the optical power P of the beam emerging from their set), attacks the front surface of the thermal sensor head. In the particular experimentation, as CO_2 laser source the LM-4 model of Edinburgh Instruments Co has been used which emits at a stabilized wavelenght of 10.6 μn, with internal water cooling, and offers the possibility to modulate its intensity - if it is desired - by means of a square-wave generator

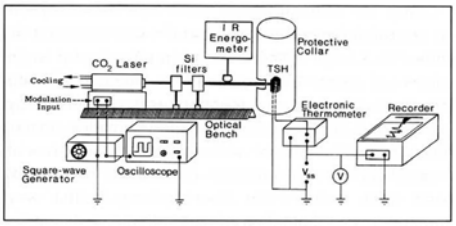

Fig. 2 - *Arrangement of the experimental set-up employed In the present work for measurements on the thermal interaction between the CO_2 laser and the material of the thermal sensor head (TSH).*

controlling straight- forwardly the current of the laser source. The amplitude of the output of the square-wave generator and the frequency of its square waveform, which is equal to the modulation frequency of the laser beam, were monitored by means of an oscilloscope continuously connected to the generator.

The nominal optical power emitted from the above laser source, in CW operation, was of the order of 350 mW, for a

temperature of the device equal to14° C ± O.5° C which has been maintained constant throughout the experiment by means of the internal closed cooling system. Nevertheless, for the purposes of our experimentation, the (mean) laser power incident upon the thermal sensor was less than the above cited value, depending on the particular combination of the attenuating Si neutral-filter plates and the frequency of intensity modulation. Therefore, the incident mean laser power was each time measured by means of an appropriate I.R. radiometer/energometer inserted as it is shown in Figure 2.

On the other hand, the thermal head, used as thermally sensitive target simulating dark biological tissue and at the same time as temperature measuring element, was a bulk PTC semiconductor thermistor of the KTY 10 type of Siemens Co. This thermistor was connected as thermally variable resistor Rϑ in the electric bridge of the electronic temperature measuring circuit shown in Figure 3. This circuit has been designed and constructed specifically for the needs of the present experimentation on the basis of design

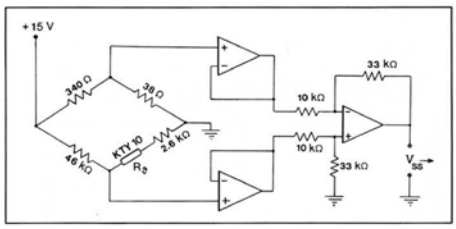

Fig. 3 - *Schematic diagram of the electronic thermometer circuit used for the KTY10-type thermal head TSH, A11 operational amplifiers are of the LM 741 type.*

suggestions of related technical literature (11-13). We have thus succeeded in implementing an electronic thermometer which is highly sensitive and speedy in response (13) with a linearity largely enhanced by the electronic circuitry of the stage, particularly for the range from O to 100°C where a technical sensitivity of about 14 mV/°C has been achieved. Note that, as seen in Figure 2, the output voltage Vss of the electronic thermometer in question is continuously monitored by means of a digital voltmeter and recorded by a chart recorder.

Lastly, the thermal sensor head TSH was fixed on an adjustable micropositioner which was sheltered as shown in a large cylindrical housing made of a white cardboard sheet. This housing was intended to keep the temperature of the thermal head unaffected by any air currents invading the experimentation space. Also, the white internal walls of the housing reemit most of the thermal radiation emitted by the thermal head while being under laser heating; in this way attempted we simulating the conditions existing in living tissues, in which - as already seen - a part of the laser energy emitted (by transmission of diffusion) from the targeted tissue region Is back-scattered or rediffused by the surrounding tissue layers (Fig. 1).

MEASUREMENTS AND RESULTS

Preliminarily, we have graduated our electronic thermometer by extracting accurately the graduation curve Vss versus ϑ, where ϑ denotes the reading in 0C of a high-precision centigrade mercurial thermometer, immersed together with our thermal sensor, head in water progressively heated up to 100°C. This curve has shown that our thermometer possesses a technical sensitivity of 13.8 mV/0C which is satisfectorily precise and constant over the 0 to 70° C range and deviates slightly for higher temperatures.

First of all, we have determined the thermo-optical step response of the thermal head, i.e., the time variation of its instantaneous temperature ϑ just after the application of a stepwise CW laser "pulse", such as the one shown by the dashed line of Figure 4. The corresponding step response curve, ϑ versus t, where t denotes time, has been extracted and represented straightforwardly on the chart recorder for various levels of the laser power P incident upon the thermal head. One representative curve of this kind, obtained for an optical power P equal to 100 mW (step value) is depicted in Figure 4. From these step response curves, the mean value of the thermal time constant (rise time) τr of the thermal head, defined as the time period needed for a rise of temperature from the 10% up to 90% of its final steady-state value, has been determined and found to be of the order of 10 min.

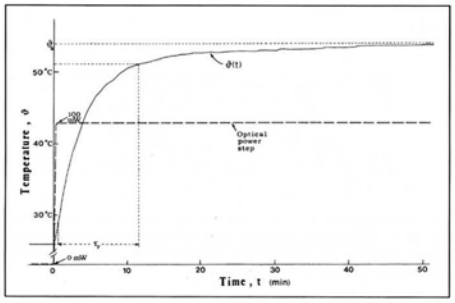

Fig. 4 - *A representative transient and steady-state response curve, ϑ(t), of the temperature ϑ of the attacked sensible material (TSH) when an optical power step of 100 mW is incident upon it. ϑ_s denotes the steady-state value of ϑ and τ_r the thermal time constant (rise time) of the material mass under consideration.*

From the same set of step response curves we have determined the dependence of the steady-state temperature rise Δϑs, produced by the beam on the thermal head, as a function of the CW-level power P of the step-shaped laser "pulse" (unmodulated) arriving at the sensor head. The so obtained Δϑs versus P curve is depicted in Figure 5, along with the maximum-error margins associated to each individual determination of Δϑs.

Next, passing to the intensity-modulation regime, we have applied to the laser beam, a full square-wave modulation by driving the corresponding input of the laser source with a train, of square-wave voltage pulses based or, a level of 0 V and with an amplitude of 11 V. According to the manufacturer's manual, an intensity modulated (IM) laser beam was then obtained; the modulation, depth was 100% and the modulation frequency fm, was equal to that of the square-wave voltage pulses (Fig. 6). Obviously, under this modulation, the mean power P of the laser pulses will be equal to the half of their maximum value (amplitude) Pm. Under these conditions, it has been ascentained experimentally that, in any case, the time temperature response of the thermal head to the above square-wave IM modulated beam has the form

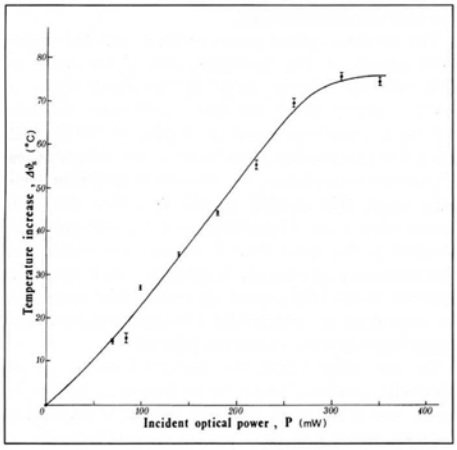

Fig. 5 - *Measured dependence of the increase $\Delta\vartheta_s$ of the steady-state temperature of the thermal head material upon the level of the (stepwise) incident optical power P of the laser beam unmodulated. The initial temperature of the thermal head was equal to 26° C.*

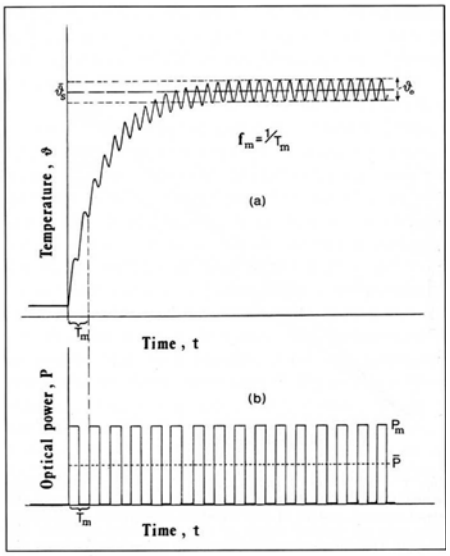

Fig. 6 - *Shape of the temperature-to-power time response (a) of the TSH material when subjected to a laser beam intensity to a laser beam intensity modulated in the square-wave form shown in (b). For the various notations see the text.*

represented in Figure 6a. As we observe from this figure, the temperature of the so irradiated thermal head, after an initial transient increase of undulating form, attains a varying steady state in which it oscillates, almost sinusoidally, about a constant steady-state mean (dc) value ϑs with an also constant peak-to-peal (ac) amplitude ϑo. To describe quantitatively the situation, it a convenient to use as principal parameter the ratio m = ϑo / Δϑs, which we shall call the temperature modulation depth.

We have monitored and recorded the time variations of the instantaneous temperature ϑ of our KTY-10 sensor head irradiated, in a stepwise manner, by various mean incident powers P of the laser beam being square-wave modulated at various modulation frequencies (repetition rates) fm ranging from 0 Hz (i.et unmodulated) up to about 0.8 Hz. From the monitored data we have derived that the dependence of the steady-state temperature increase Δϑs on the mean power P of the laser beam, for various modulation frequencies fm, is described by the curves given in Figure 7.

In addition, from those of the above recordings which concern a standard incident mean power of 100 mW we have extracted the corresponding variations of the amplitude ϑo of temperature oscillations and the temperature modulation depth m = ϑo / Δϑs with modulation, frequency/repetition rate fm. These variations are plotted in Figure 8 by the curves a and b, respectively, along with the corresponding depend¬ence of the temperature increase Δϑs on the frequency fm (curve c).

Lastly, from the same recordings it has been ascertained that the mean time constant (thermal rise time) of the sensor head does not undergo but a slight decrease with increasing fm and continue to be of the order of 10 min.

ANALYSIS AND DISCUSSION OF RESULTS

First of all, as seen from Figure 5, in the case of an unmodulated laser beam the increase Δϑs in the steady-state temperature of the irradiated material (which, in our case, is simulated by the medium of the thermal sensor head) proved to be an increasing but non-linear function of the step-wise incident optical power P of the laser light stream. Moreover, as seen from the figure, at higher optical powers the nonlinearity in question turns particularly marked and the Δϑs – P curve enters a characteristic saturation region and tends to become horizontal; hence no additional temperature increase is further possible, no matter how much we further increase the incident laser power. This saturation effect should be attributed to thermal compensation phenomena, such as the balancing between the light energy absorbed by the thermally sensible mass and the amount of energy re-emitted by thermal radiation, while taking into account the dispersion of energy due to the scattering processes quoted in the 2nd section (Fig.1).

Anyway, in practice the foregoing nonlinearity and saturation features of the Δϑs – P curve may be the cause of non-negligible complications, particularly when the laser beam system is used for treatment or biomedical purposes. In effect, as a result of these features, it is difficult and/or unreliable to try controlling successfully and accurately the laser beam power by means of the output of the electronic thermometer attached to the termal sensor head. This drawback is even more bothering in the case of "smart laser" medical systems that maintain the temperature of the attacked tissue at a desired constant value by continuously monitoring the tissue temperature, via a thermal head embedded in the tissue, and correspondingly readjusting the incident power P by means of an automatic control system. In such systems, which are necessary for achieving a predetermined therapeutic result (coagulation, ablation, etc) as cited in the 2nd section, a good linearity of the temperature-power dependence is of crucial importance and therefore the nonlinear and saturated form of the curve of Figure 5 may be a noticeable source of error or malfunction. On the other hand, the KTY 10 thermal head (thermistor) used in the present experimentation is made of a bulk bakelite-type material (mixture of cobalt, nickel and other oxides (13) of black colour and therefore - according to what has been cited in introduction — can be considered, even roughly, as equivalent to dark biological tissue subjected to CO_2-laser radiation. On this condition, the foregoing result on saturation effect implies that at least when dark

tissue is irradiated by a CO_2 laser beam (unmodulated), it is impossible to overcome a maximum possible tissue temperature ϑm no matter how strongly we increase the incident power of the laser beam. Presumably, this maximum temperature m could be increased by changing other physical parameters of the beam such as the angular aperture (by means of a focusing lens), the wavelenght, etc.

Passing now to the case of intensity-modulated laser beam, we first observed that, as shown in Figure 6, the square-wave modulation of the light intensity of the beam is transformed into an almost sinusoidal modulation of the temperature of the targeted material. This effect should be attributed to the thermal inertia of the considered mass of sensor material which, as seen, exhibits a thermal time constant Ta of the order of 10 min, hence much larger than the highest period Tμ used in our experimentation, which is Tμ =1/0.07 Hz ~14 sec.

Further, from inspection of the curves of Figure 7 we deduce that the application of intensity modulation to the laser beam suppresses drastically the drawbacks of nonlinearity and saturation observed in

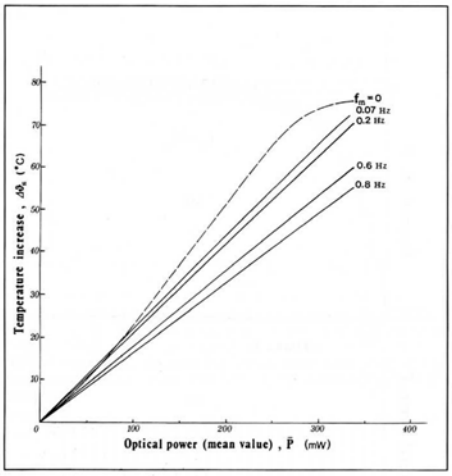

Fig. 7 - *Variation of the increase $\Delta\vartheta_s$ in the steady-state temperature of the thermal head material as a function of the mean optical power of the incident modulated laser beam, measured for various values of the modulation/repetition frequency. The curve for the unmodulated beam (dashed line) has been traced from Figure 5 for comparison purposes.*

the temperature-to-power curve of the unmodulated beam, although this suppression is paid with a corresponding lowering of the temperature increase induced by the beam. Note, also, that the higher the modulation frequency fm the higher the as above enhancement of linearity is. Anyhow, as derived from the figure, in the present case this linearity enhancement is practically complete from a frequency of the order of 0.1 Hz, hence for a repetition period less than 10 sec. In consequence, in case the thermal sensor head under investigation is used as part of an automatic control system for stabilizing temperature in irradiated tissues it would be our interest to modulate the laser light with a modulation frequency (repetition rate) as higher as possible, provided that the Δϑs – P response of the head would be not inacceptably diminished.

On the other hand, the above imply that, in a real living tissue of dark colour with a behaviour similar to that of the present thermal head, even a modulation frequency of the order of 0.1 Hz, which is moderate in value, would be enough to suppress the temperature saturation effects in the tissue without limiting noticeably the achievable temperature levels.

Lastly, from inspection of the curves of Figure 8 it is first deduced that the amplitude and/or the modulation depth of the temperature oscillations provoked by an IM modulated beam of constant mean power P decrease with increasing repetition rate fm, so that practically no temperature modulation subsists after a cut-otf value fc of about 0.3 Hz (Curves a and b in Fig. 8). By contrast, as seen from Figure 7 and more clearly from curve c of Figure 8, the correspondingly induced increase in the steady-state temperature of the attacked material decreases with frequency but so slightly that, even roughly, it can be considered practically constant, particularly at higher frequencies (repetition rates). The applications of these results in the medical field have been already studied experimentally in the case of a Nd:YAG laser by other researchers, which have found that the pulsing rate of the laser beam does not influence the tissue temperature profile or the nature and extent of the tissue damage (20). Nevertheless, it is worthy noting that the degree of the temperature modulation under discussion,

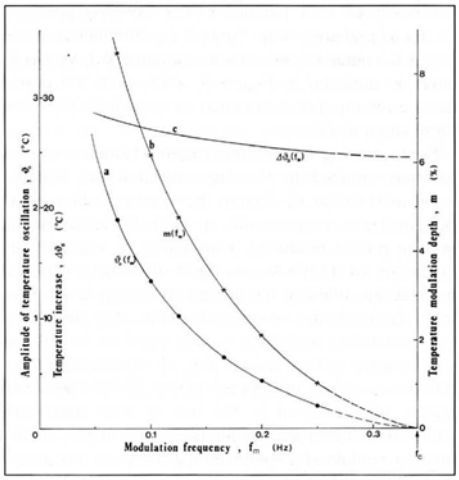

Fig. 8 - *Variation of the amplitude ϑ_o of the steady-state temperature oscillation (curve a), the corresponding temperature modulation depth m (curve b), and the steady-state temperature increase (curve c) $\Delta\vartheta_s$ as functions of the modulation frequency f_m of the modulated laser beam, measured for a mean optical power $\bar{P}$ = 100 mW.*

though not particularly affecting the steady-state temperature conditions, may play a non-negligible complementary role in questions such as the smoothness and precision of the cutting contour, the range and extent of heat intrusion to the adjacent layers, etc. Accordingly it would be useless te try influencing these secondary effects by means of repetition rates exceeding the cut-off frequency fc above which- as seen- the temperature modulation of the tissue becomes negligible.

SUMMARY AND CONCLUSION

Throughout the paper the thermal (temperature) response of a bulk thermal sensor head, such as the KTY 10 thermistor, to a CO_2 laser beam has been experimentally studied with the beam, being in a CW and in an IM-modulation regime as well. Also, the corresponding influence of the (mean) optical power P and the modulation frequency (repetition rate) fm on the steady-state temperature s of the targeted mass has been investigated in detail. The results of this investigation have been evaluated and interpreted with the thermal head considered as well as thermometric sensor as simulation of a living tissue.

We have thus ascertained that the application of intensity modulation, in a square-wave format, may ensure a considerable linearization of the temperature

increase versus optical power curve and suppress the bothering saturation effect appearing in the case of unmodulated beam. This influence of IM modulation is accompanied by an undesired decrease of the temperature increment, but this effect is rather weak and may be considerably reduced by choosing a small or even moderate modulation frequency fm (in the present case, of the order of 0.1 Hz). In accordance to the above, the IM modulation of the beam can be employed either for improving the performance of an automatic control system commanding the beam-induced tissue temperature or for liberating the attacked tissue from the temperature saturation drawback.

In addition, it has been found that the intensity modulation of the laser beam is transformed into a modulation (oscillation) of the correspondingly indu ced steady-state temperature only for repetition rates fm lower than a cut-of frequency fc and the so obtained temperature modulation depth m decreases when fm increases. This fact, which has been also observed when a Kr-ion laser was used by the first of the authors et al, imposes employing repetition rates clearly lower than fc whenever a strongly modulated steady-state tissue temperature is required in medical laser applications.

In conclusion, the intensity modulation of a laser beam lowers (to a small extent only) the level of the achievable temperature increase of the irradiated material but offers very useful advantages such as the enhancement of the temperature-to-optical power linearity, the suppression of saturation effects, and possibility for modulation of the steady-state temperature. Obviously, direct investigations of the above in pure biological tissues would be particularly useful.

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Keywords: Endogenous fluorophores, autofluorescence, spectroscopy, cell metabolism, tissue organization histological structure (organization), diagnosis.

Autofluorescence Spectroscopy for diagnosis: from cells to tissues.

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ABSTRACT

Cells and tissues, upon excitation at suitable wavelengths, give rise to a fluorescence emission – autofluorescence – due to the presence of endogenous fluorophores. Autofluorescence emission properties are closely dependent on the amount, topological distribution and microenvironment of the endogenous fluorophores, in strict relationship with the metabolic processes and the histological organization in which they are involved. Alterations of the physiological state or rising of pathological conditions result in changes of biochemical composition and/or structural organization of the biological substrates, that can affect the autofluorescence emission properties. As a consequence, analysis of autofluorescence signals can be exploited to obtain information on the morfo-functional conditions of the biological substrate, suitable for diagnostic purposes.

The endogenous fluorophores mainly responsible for the autofluorescence emission of cells and tissues are here briefly described, along with an overview of diagnostic applications, mainly as to our experience is concerned.

INTRODUCTION

The presence of biomole cules acting as endogenous fluorophores makes the biological substrates able to give rise to a fluorescence emission in the UV - near IR spectral range when exposed to irradiation at suitable wavelength. This form of fluorescence is called "autofluorescence", "naturally-occurring fluorescence" or "light-induced fluorescence (LIF)".

Autofluorescence is generally much more intense in the biological material belonging to the vegetable kingdom than to that of the animals. In fact, plant tissues naturally contain a large variety of fluorochromes that, for their spectral features and quantum efficiency, find wide application as markers for cytofluorometric analysis. Examples are found among derivatives such as quinones, coumarines, cyanines, tetrapyrroles and many alcaloids. In this relation, natural fluorescence has always been considered a powerful tool in the study of vegetables morphology and physiology [1]. It cannot be said as much for autofluorescence in animal tissues. The low spectral specificity and the quite decreased quantum yield result in the fact that, for a long time, autofluorescence was mainly a nuisance for most biological fluorescence microscopists, as it may mimic some specific induced fluorescences or it contributes to reduce the Signal-to-Noise ratio of the measurements.

In spite of such a drawback, autofluorescence was the first kind of fluorescence which was investigated by microscopy. Sutro and Burnam [2] reported different autofluorescence properties in normal and pathological tissue under UV irradiation in 1933. Similar results were reported by Herly in 1944 [3], who attempted to correlate variations in colour emission with benign and malignant macroscopic organ structures. Autofluorescence microscopy was reviewed in detail by Haitinger [4].

At present, the technological improvements in the fields of excitation sources, light delivery systems and devices for the detection and analysis of fluorescence signals, together with a better knowledge of the photophysical features of the endogenous fluorophores, make autofluorescence spectroscopy a very promising approach for the characterization of biological animal tissues.

The important role of the endogenous fluorophores in the most part of the biological processes makes the autofluorescence spectroscopy a very promising tool in the study of the morpho-functional conditions of cells and tissues.

ENDOGENOUS FLUOROPHORES

The number of biomolecules that can act as endogenous fluorophores in the biological tissues is very large. The diagnostic meaning of endogenous fluorophores derives from their involvement in cells and tissue histological organization, and/or from their role in metabolic pathways. A brief description is here given with particular reference to the endogenous fluorophores the emission properties of which are favourable for investigation under microscope or via fiber optic probes. Their excitation/emission properties are reported in Table I.

The fluorophores involved in cell and tissue structure and architecture organization are

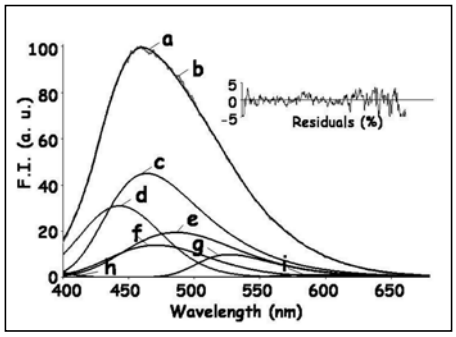

Fig.1. Curve fitting analysis of spectra recorded from bulk liver tissue. Curve (a) refers to the measured spectrum, and curve (b) is generated by the fitting analysis. The curves referring to the spectral functions describing the spectral profile of the single fluorophores, and the related parameters (peak center wavelength, λ; Full Width at Half intensity Maximun, FWHM) of fitting, are: (c) free NAD(P)H (λ = 463 nm; FWHM = 115 nm) and (d) bound NAD(P)H (λ = 444 nm; FWHM = 105 nm); (e) Vitamin A (λ = 488 nm; FWHM = 102 nm); (f) Arachidonic Acid -Fatty acids- (λ = 470 nm; FWHM = 90 nm); (g) Flavins (λ = 526 nm; FWHM = 81 nm); (h) Proteins (λ < 440 nm); (i) Lipopigments (λ =587 nm; FWHM = 80 nm).
Residual analysis shown at the bottom of each spectral presentation. Fitting r^2 coefficient of determination = 0.997.

mainly related to the proteins, because of the presence of the aromatic aminoacid tyrosyne (Tyr), phenylalanyne (Phe) and thryptofan (Trp). According to the kind of aromatic amino acids present, proteins can be classified as class A (Tyr, Phe) and class B (Trp, Tyr, Phe). The fluorescence emission of proteins depends on the amino-acid composition, and can be influenced by the primary structure, the spatial conformation and the microenvironment properties. Actually, tryptophan-containing proteins show excitation and emission spectra different from those of the pure amino acid, depending on the microenvironment occurring in the protein chain [5-7]. Generally proteins are characterized by absorption and emission bands in the 240 – 300 nm and 300 – 350 nm spectral ranges, respectively.

This is the case of the globular proteins such as enzyme, albumin, immunoglobulins [6].

The structural proteins collagen, elastin and cytokeratins , belonging to the fibrous protein class, differ from this general behaviour, being characterized by both a shift to longer wavelengths and an increase of the quantum efficiency of the emission, that make them suitable for fluorescence microscopy investigation.

Collagen is the major extracellular matrix component. Its fluorescence properties are generally associated to both to the presence of hydroxylysyl pyridinoline and lysyl pyridinoline [8], and the formation of covalent cross-links, directly related to the age and degree of maturation [9, 10]. At least 11 types of collagen are ranked, according to the composition of the monomeric chain and the degree of polymerization [11], although 80 - 90 % of the collagen in the body consists of types I, II, III.

Elastin is the major component of the elastic fibers and is found in most connective tissues along with collagen and polysaccharides. Its fluorescence is attributed to the presence of a tricarboxylic triamino pyridinium derivative, a residual confined in proteases-resistant regions [12]. Cytokeratins are structural, intracytoplasmic keratins, typically present in epithelial cells. They increase during the active phase of tumour growth, and play an important role as tumour markers [13]. Lipids in general do not contribute greatly to tissues autofluorescence since only few of them are fluorescent with a rather low efficency. Among them, phospholipds and their oxidation products [6], and arachidonic acid, a fatty acid precursor of prostaglandins are reported. [14]. Triglycerides, the form in which most fat exists in the body, are not fluorescent. They may accumulate to different amounts because of an altered metabolism or as the result of catabolic reactions , giving rise to the occurrence of intracellular droplets, that can exhibit a noticeable fluorescence emission due to the presence of liposoluble fluoresceing compounds, such as vitamin A and drugs.

Lipopigments are a class of heterogeneous macromolecules composed by lipidic chains, aggregated with varying amounts of proteins, glycoproteins, carotenoids and melanin-related compounds. The chemical complexity of lipopigments accounts for their fluorescence properties variability [15]. Lipopigments are naturally associated to cell ageing process [16]. The products accumulating during ageing are generally called lipofuscins, while those related to pathological processes are called ceroids, although they share both some physicochemical properties and some steps of their biochemical and structural rearrangements. In ophthalmology, an increasing interest is paid to the presence of lipofuscins in the retina. The abnormal increase in the autofluorescent pigments accumulating as lipofuscins in the retinal pigment epithelium has been indicated to play a role in the cell function decline [17]. On this base, fundus autofluorescence analysis is currently considered as a novel imaging method for both investigation and diagnosis of various hereditary and complex retinal diseases [18].

Pyridine nucleotides and flavins are coenzymes closely involved as major electron carriers in several metabolic, anabolic and catabolic pathways occurring in the cell cytoplasm and account for the most part of autofluorescence signal rising from this cell region [19]. Pyridine nucleotides are fluorescent in the reduced form. NADH - generated via glycolysis and Krebs cycle - is eventually oxidized to produce ATP, while NADPH - generated by the penthose phosphate pathway – act as an electron donor for reductive biosyntheses and cell antioxidant defence.

NADH and NADPH do not show appreciable differences in the spectral properties. NAD(P)H is present in both free and enzyme-bound forms, the latter being expression of the active involvement of the coenzyme in the energetic metabolism. Binding to enzyme molecules results in a blue-shift of the NAD(P)H emission peak position [5], an increase by about 3 times in the quantum efficiency of NAD(P)H [20] and a lengthening of fluorescence decay time (1.0 ns for the bound forms vs 0.4 ns for the free forms) [21].

Flavins are fluorescent in the oxidized state. The excitation /emission properties of the free forms of both flavin mononucleotide (FMN) and flavin-adenin dinucleotide (FAD) in aqueous solution (pH 7) are very close. The fluorescence properties of FAD are strongly affected by the nature of the protein to which the prosthetic group is

bound. In the cells, only the flavoproteins as lipoamide dehydrogenase and electron transfer flavoprotein in the mitochondrial matrix seem to contribute significantly to cellular flavoprotein fluorescence [22, 23].
Among vitamins, Vitamin B6 and A deserve attention for their fluorescence properties and functional metabolic engagement in tissue under healthy and altered conditions. Vitamin B6 (Pyridoxine) and related compounds are diffused in all the tissues, and take part to a great number of reactions concerning metabolism of amino acids and, likely, of lipids. Autofluorescence of Pyridoxine and its metabolites, however, is rarely considered: difficulties to discriminate it from the emission of proteins, such as collagen or elastin, or of NAD(P)H have been reported [24].
Vitamin A (Retinol VI) is characterized by an appreciable emission signal, already reported by Querner in 1932 [25], and subsequently studied by Popper [26]
both in human and in rat. Vitamin A fluorescence properties have been already considered for serum assays [6, 27]. Particular attention has been given to vitamin A fluorescence in the liver, the organ mainly responsible for its storage, metabolism and mobilization to the plasma in order to provide for the organism needs. The strong photolability of vitamin A was exploited to eliminate its signal during UV irradiation when liver energetic metabolic studies based on NAD(P)H emission analysis were performed [28, 29]. On the other hand, evidences are rising about both the relationship between vitamin A and lipids in the liver tissue, and its implications in altered metabolic conditions and pathology progression [30, 31]. In this context, vitamin A can be positively considered, as an indirect marker of lipids, for developing of a diagnostic procedure to investigate hepatic metabolism in normal/altered physiological conditions and disease progression, based on its photolability [32, 33].
Among endogenous porphyrins, protoporphyrin IX is the tetrapyrrolic ring ultimate precursor during biosynthesis of Heme. Porphyrins are ubiquitous molecules that, after complexation with Fe, ions, participate to a variety of biological processes, such as transport of oxygen, catalysis and pigmentation. In the absence of metal complexation, they can give rise to a typical fluorescence emission in the red region.
Accumulation of porphyrins can occur as a consequence of defects in biosynthesis of heme, resulting in a pathology (porphyrias) with an usually associated photosensitivity [34]. A spontaneous and occasional presence of fluorescence signals in the red region emission has been found when performing spectrofluorometric studies of neoplastic masses [35-40]. Different explanations have been proposed for this phenomenon, such as the presence of bacteria in ulcerated and necrotic tissues [35], or changes in the activity of enzymes associated with heme biosynthesis in peripheral blood mononucleated cells [41, 42], and altered metabolism of heme and iron [43] in tumour bearing subjects.
The neurotransmitters, or biogenic amines, including derivatives from catecholamines (dopamine, adrenaline and nor-adrenaline) and from serotonin (5-hydroxytriptamine, 5-HT) are fluorescent in the UV region 5-HT can undergo both oxidation and dimerization/trimerization processes leading to a shift of the absorption/emission spectra to wavelengths longer than those of the native ones. These conditions favor fluorescence spectroscopy investigations via fiber optic probe thus opening interesting perspectives for an in vivo evaluation of the response of neurotransmitter level to pharmacological treatments by means of autofluorescence [44].

DIAGNOSTIC APPLICATIONS OF AUTOFLUORESCENCE

The overall autofluorescence emission of cells and tissues is strictly dependent on the chemical nature, the amount, the spatial distribution and the microenvironment of the fluorophores in the biological substrate. The endogenous fluorophores are associated to a variety of biomolecules that can be either responsible for the structural arrangement or involved in the metabolic and functional processes of cells and tissues. Proteins, such as collagen, elastin or more generally constitutive proteins belong to the former case pyridinic coenzymes (NAD(P)H), flavins, riboflavin, pyridoxine derivatives, porphyrins, in addition to accumulation products of catabolic processes such as lipofuscins and lipopigments, belong to the latter case. Changes in the morphological and biochemical properties of cells and tissues related to physiological state or induced by the occurrence of pathological processes are expected to modify both amount and distribution of the endogenous fluorophores thus affecting the autofluorescence properties. On this basis, autofluorescence, as an intrinsic parameter of biological substrates, provides the basis for a real time, in situ monitoring of the morphofunctional conditions of living cells and tissues, in the absence of administration of exogenous markers [45-51].
When the spectral parameters of pure compounds corresponding to the endogenous fluorophores present in a cell or tissue are defined, they can be exploited to perform a fitting analysis of the overall emission signal, allowing the estimation of the relative contribution of each single fluorophore, similarly to a biochemical analysis (Figure 1).

a. Application at cell level.

Studies performed on cultured mammalian cell lines demonstrated that autofluorescence of living cells under excitation at wavelengths longer than 340 nm originates in the cytoplasm and is almost absent from the nucleus. Comparison of spectra recorded on cells with those of known cellular components suggested that most of the cell fluorescence arises from the energy metabolism-linked fluorophores nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, and flavin coenzymes [19, 52, 53]. Simultaneous monitoring of the redox state of these coenzymes is reported to be

Table I -Endogenous fluorophores

Fluorophores	Excitation (nm) (peak position range)	Emission (nm) (peak position range)
Aromatic Amino Acid Residues Proteins: class A= Phe+Tyr; class B= Trp+Phe+Tyr	240 – 280	280 – 350
Structural proteins		
Extracellular:		
Collagen	330 – 340	400 - 410
Elastin	350, 420	420, 510
Intracellular		
Cytokeratins	280, 325	495, 525
Reduced Pyridine Nucleotides NADH and NADPH	330 – 380	440 (bound) 462 (free)
Flavins		
Riboflavins, FlavinMonoNucleotide (FMN), prostetic groups of flavoproteins	350 – 370 440 – 450	480 – 540
Porphyrins (Protoporphyrin IX, Zinc porphyrin)	405 500 – 600	630, 670 (590, 640)
Lipofuscins. Lipopigments	UV 400 – 500	> 540
Vitamins		
Vitamin A	370 – 380	490 – 510
Vitamin B6 and precursors	290 – 310 / 375 – 395	375 – 395 / 400 – 500
Lipids		
Arachidonic acid	330, 350	470 – 480
Phospholipids	430 - 440	520 – 570
Catecholamines:		
Adrenaline, noradrenaline and dopamine	280 – 290	320 – 340
Serotonin	305, 360 (dimer), 420 (trimer)	350, 440 (dimer), 520 (trimer)

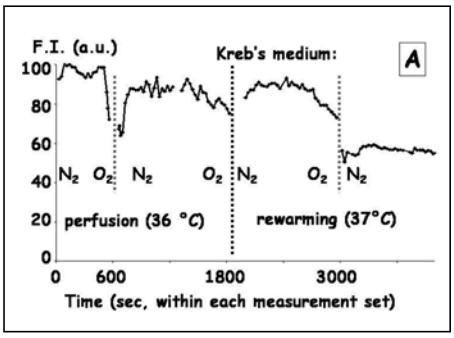

Fig. 2. (A, B) Autofluorescence emission monitoring in a Wistar rat liver. Excitation (366 nm) and emission light are respectively delivered and collected by means of a single fiber optic probe. The instrumental arrangement was already described in detail (80). The liver is explanted, washed and submitted to perfusion with oxygenated Kreb's medium, mimicking the transplantation-preservation phase. The functionality of liver energetic metabolism is investigated by means of a cycling induction of hypoxia, and evidenced according to the emission signal changes. This increase, reflecting the ability to accumulated NAD(P)H in the reduced form under hypoxic condition, and decreases upon reoxygenation. Sequences of brief anoxia (Kreb's saturated with N_2), followed by reoxygenation are applied at interval times of about 1 h during liver perfusion with Kreb's medium at 36 °C (A) and 18 °C (B). The signal changes are much greater for (A) than for (B). The latter maintains the ability to respond to N_2 also at the end of perfusion, during the rewarming phase (perfusion with Kreb's medium at 37°C). The results evidence an increase in tissue metabolic with the increasing of the Kreb's medium temperature.

expression of energetic metabolic state in several biological models, in both physiological and experimental conditions [45, 54]. The early investigations performed by Britton Chance showed changes in autofluorescence emission of intact cells in relation with the aerobic-anaerobic condition, that were explained in terms of an alteration of the redox state of the coenzyme NAD(P)H [52, 53]. These pionieristic studies opened the way for a lot of works on the NADH fluorescence aimed to study energetic metabolism engagement in intact mitochondria, living cells and tissues, under physiological and altered condition [55-58]. Additional information is likely to be provided by pyridine nucleotides analysis, considering also their roles as signal transducers, and the vital importance of NADP in the cell oxidative defence and in reductive biosynthesis [59].

A dual information can be given by redox fluorometry: 1) the signal amplitude, which reflects the amount of NAD(P)H in the reduced form, and that can be considered, for example, to investigate the cells or tissues capacity to respond to changes in oxygen availability; 2) the spectral shape, which reflects the ratio between free and bound forms of NAD(P)H, and is significant of the prevalence of anaerobic or aerobic energetic metabolism.

Differences in the equilibrium between reduced and oxidized forms as well as between the free and bound reduced forms of the pyridine nucleotides were found, for example, when comparing cultured cells in different growth conditions [60, 61], isolated cells from normal and tumor tissues [62], cells during prolonged hypoxia [63] and leukocyte families [64]. As to this latter case, spectroscopy and imaging investigations allowed to discriminate normal white blood cells on the base of their autofluorescence emission properties, which, in turn, were ascribed to differences in the intrinsic metabolic engagement rather than on cell dimensions. A direct relationship between autofluorescence properties and morphological and functional rearrangement of cells structures was further demonstrated to characterize the maturation steps of leukemic cell models upon induction of differentiation [65].

Application of this analytical procedure evidenced a relationship between NAD(P)H autofluorescence and the neoplastic condition in cultured populations of normal and transformed fibroblast, derived from syngeneic rats [20, 66]. A red shift of NAD(P)H emission spectrum was found indicating an increase of the coenzyme free form, according to the prevalence of NAD(P)H free form occurring in

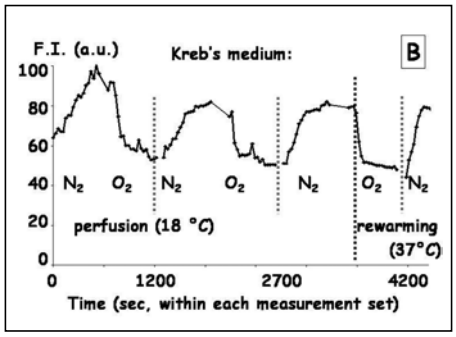

Fig.3. Tissue autofluorescence spectra (exc 366 nm) recorded in vivo, via fiber optic probe during endoscopy, from non-neoplastic and diseased colonic mucosa. The amplitude of the emission signal recorded from adenoma and adenocarcinoma is lower than those recorded from the respective non-neoplastic mucosa of the same patients. The emission signal from carcinoma is lower and red shifted with respect to that of adenoma. Similarly, the non-neoplastic mucosa of the patient bearing a colon carcinoma, shows an emission signal lower and red shifted than that of the adenoma- non-neoplastic mucosa. An occasional presence of a well defined iband at 630 nm, likely corresponding to porphyrins is found in the carcinoma.

transformed cells. This finding was attributed to the lowering in the binding sites for NAD(P)H in the cancerous tissue, according to the decrease in mitochondrial activity and density [20, 62, 66, 67]. Autofluorescence analysis, therefore, provides evidences in agreement with data reported in the literature [68], showing that changes in the cell energetic metabolism occur that and depend on the acquired status of malignant phenotype.

In the single cells, lipopigments are additional fluorophores likely contributing to autofluorescence. Lipopigments are a heterogeneous group of substances, characterized by a yellowish fluorescence emission that are supposed to derive from lysosomial material through oxidation and polymerization processes. These pigments appear usually to be confined in brightly fluorescent cytoplasmic granules and have been found to modify their amount and properties in relation to ageing processes [16] and to their functional engagement [69] alues derived from intraneuronal lipopigment emission spectra from Kufs' desease [70] and Sanfilippo syndrome [71] could be distinguished from those obtained from non-diseased brain.

b. Application at tissue and organ level.

Autofluorescence properties of a tissue must take into account that biological material acts as a turbid medium. Therefore, excitation and emission properties result from three components: i) the tissue optical properties (absorption and scattering coefficients) at the excitation wavelength, which determine diffusion and distribution of excitation light within tissue; ii) the emission characteristics of the endogenous fluorophores at different depth within the tissue, which results from the fluorescence quantum efficiency of the fluorophore and the excitation intensity at that depth; iii) the tissue optical properties at the emission wavelengths, which determines the overall fluorescence escaping the surface of the tissue.

The concentration and distribution of non-fluorescent absorbers and scatterers within the tissue will thus affect the propagation of the light (both excitation and emission) influencing the signal amplitude and the spectral shape of the autofluorescence at the site of signal collection. The excitation light will be of great importance, since an inverse relationship occurs between penetration depth in a biological tissue and wavelength. In this view, the autofluorescence properties of a tissue can be related to both its biochemical and histological features that, in turn, define the nature and the intratissue distribution of the endogenous fluorophore, respectively. Alterations of both structural organization and metabolic activity of tissue, taking place as a consequence of pathology occurrence, can thus affect the autofluorescence signal properties. A typical example is provided by the autofluorescence properties of the bulk cerebellum tissue of rat during postnatal development. A mild cytotoxic treatment with the anti-tumoral drug cisplatin was used to purposely affect the rearrangement of the multilayered architecture of the cerebellum cortex. Differences in autofluorescence properties occurred between treated and untreated rats, that were attributable to both biochemical and histological organization [72].

In bulk tissues, autofluorescence analysis can be applied for: i) characterization and monitoring of metabolism under normal and physiologically or purposely altered conditions; ii) diagnosis of pathologies.

As to metabolic monitoring, experimental works have been performed on the response of organs to ischemic conditions and on the subsequent recovery ability.

Heart, kidney and liver are the organs mainly considered for the development of procedures for the monitoring of their functional conditions [73-77]. A very recent application has been also proposed on the base of the correlation occurring between changes in autofluorescence spectra and the rejection grade of transplanted hearts, for the detection and monitoring of heart allograft rejection [78].

In clinics, to respond to the necessity of surveying organs vitality, a device has been developed for the contemporary monitoring of parameters significant of tissue vitality, that is blood circulation and oxygen balance, along with an autofluorescence amplitude signal attributed NADH. [79].

Several studies have been performed on the liver, to set-up procedures for the monitoring of its functionality during experimental transplantation phases, with the aim to improve its preservation conditions [80-81].

Our experience on rat models evidenced that the dynamic response of the tissue autofluorescence to an external stimulus purposely applied, such as oxygen availability, can provide multiple information about the engagement of metabolic pathways in livers under different experimental conditions. The kinetics of the decrease in the signal amplitude occurring when an hypoxic phase is followed by reoxygenation, relies mainly on the amount of NADH, and on its direct involvement in oxidative respiratory processes (Figure 2). When compared with the respective controls, slower kinetics were found for livers submitted to purposely damaging treatments mimicking transplantation

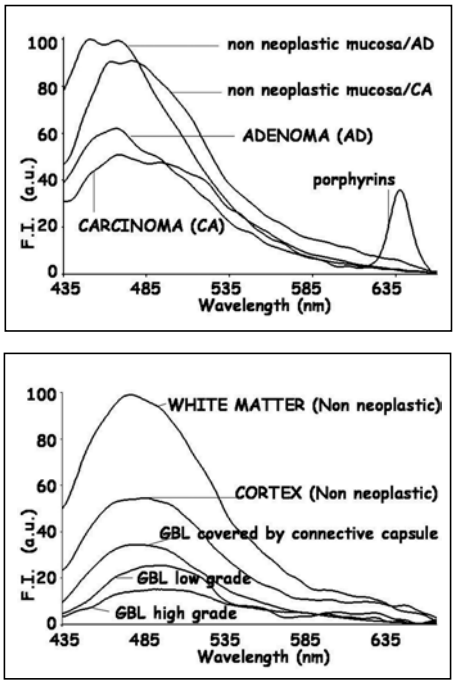

Fig.4. Tissue autofluorescence spectra (exc 366 nm) recorded *in vivo*, via fiber optic probe, during surgical operations on healthy tissue (cortex and white matter) and on glioblastoma. Although normal cortex shows an emission signal lower than that of the white matter, much lower autofluorescence signals are obtained from low and high grade glioblastoma, even in a case when the lesion is covered by a capsule. The autofluorescence spectra recorded from neoplasia show also a shift towards longer wavelengths when compared with those of the non-neoplastic tissues.

phases, reflecting the impairment of the mitochondrial respiratory chain [82]. On the contrary, a the uncoupling effect on oxidative phosphorilation induced by thyroid hormones, facilitating the energy transduction pathways, resulted in a faster kinetics in hyperthyroid rats. On the other hand, the hypermetabolic conditions of hyperthyroid rats resulted in an autofluorescence signal amplitude higher than in normal rats, ascribed to an increased presence of NADPH and thus associated to metabolic functions not directly related with respiratory processes [83].

As to the pathology diagnosis, one of the most explored applications of autofluorescence spectroscopy concerns the detection of neoplastic growth. Already in 1943 Herly [3] attempted to make a differentiation between benign and malignant lesions observing the different "specific colours" rising from breast specimens under filtered UV light. Those observations were made soon after excision, with the aim to determine the extent of the tissue removal during surgical operation. The quantitative fluorescence spectroscopy for the discrimination of normal and malignant tissues was further supported by the work of Lycette and Leslie [84]. These authors found that the fluorescence intensity of excised cancerous tissues were less than those of normal tissue from the same patient, and suggested that it could be due to tumor necrosis. Differences in the spectral profiles of both normal and cancerous tissues, as well as a blue-shifting of the emission peak in the spectrum of malignant with respect to normal tissue upon excitation at 488 nm were then reported in animal model by Alfano et al. [85] who attributed the tissue fluorescence to flavins and porphyrins, and justified the differences on the basis of different environments in the two types of cells. Yanlong et al. [86] reported that under excitation at 365 nm cancerous tissue exhibited emission peaks at 630 and 690 nm that were not observed in the spectra of normal tissues and were attributed to naturally occurring porphyrins.

Based on these early works, several groups have investigated extensively the use of autofluorescence spectroscopy for the diagnosis of malignant and premalignant tissues both in vitro and in vivo. The guiding of light via fiber optic probes opened the possibility of application whenever a measurement can be performed at direct-contact with the "suspect" sites. The instrumental set-up for autofluorescence measurements in vivo was in general derived by adapting that used for cancer diagnosis based on administration of exogenous porphyrins. Autofluorescence analysis is performed on the base of both spectroscopy and imaging techniques [87-91], and can be applied to tissues either exposed or accessible via endoscopy.

At present, most of the anatomical sites is investigated by means of autofluorescence for diagnostic purposes. Some of the more recent examples of literature are here summarized in relation with organs and tissues investigated: gastro-intestinal tract - that is oral cavity [92 - 94], esophagous [95], stomach [96-98], colon [99, 100] - lariynx [101, 102], and bronchus [103 - 105], breast [106, 107], cervix [108 - 110], bladder [111, 112], brain [113 - 117], head and neck [118], skin [119, 120], bile duct [121], metastatic limph nodes [122].

Light-induced fluorescence endoscopy (LIFE) diagnostic systems based on the ratio analysis of autofluorescence images collected simultaneously form a defined mucosa surface area in the green and red emission regions, have been developed by Xillix (Novadaq Technologie Inc., Missisauga, Ontario, Canada) with particular relation to bronchial and gastro-intestinal mucosa. Autofluorescence analysis can be of great help to find early lesions, otherwise difficult to be found under normal white light observation in the case of epithelial tissues, such as the bronchi [123]. In the colon it can allow the detection of suspicious tissue areas when collecting biopsies from colonic mucosa, such as in the case of flat displasia [99].

Here we report of two examples of autofluorescence diagnosis concerning our experience on colon and brain, that represent two different kind of tissues, that is multylayered and non-multilayered tissues.

In the case of the colon - formed by multiple layers of epithelial cells and intercellular material, and of connective tissue- the invasiveness of neoplasia results in the sinking or even in the disappearance of the submucosa, that is replaced by the proliferating neoplastic cells and by their loose connective. As a consequence, the contribution of brightly fluorescing constituents of the normal submucosa, collagen and elastin, is strongly decreased or lost, resulting in a reduction of the fluorescence emission amplitude recorded from the surface of the tissue [39-124-127]. This decrease in signal emission is accompanied by changes in the spectral profile (Figure 3). A Monte Carlo model was developed to simulated the fluorescence emission from the tissue, assigning the autofluorescence properties measured on specimens ex-

vivo, to each of the three or two layers assumed to compose the normal and the cancerous tissue , respectively. The different modelling of morphology and histological arrangements indicated that the differences in autofluorescence emission between normal and neoplastic tissue was to be ascribed to the modification in morphology accompanying the neoplastic growth, rather than to the differences in the endogenous fluorophore nature [128].

In the case of brain tumours, namely glioblastoma, the autofluorescence properties of non-neoplastic and neoplastic tissues were analyzed both on tissue sections and homogenates, by means of a microspectrofluorometer, and on patients, during surgical operation, with a fiber-optic probe [113, 114]. The aim was to develop an autofluorescence-based diagnostic technique that can be applied easily to guide brain tumor resection during neurosurgical operation. A broadening of the emission band along with a red shift of the emission peak and a signal amplitude decrease were found for the glioblastoma lesion autofluorescence in comparison with those of the healthy tissues, cortex and white matter (Figure 4). These changes could be explained according both to a decreased contribution of NAD(P)H, in agreement with previously published data [129], and with a favored presence of the NAD(P)H free form. These findings were consistent with the shift towards anaerobic metabolism of the neoplastic condition [66, 130]. The fact that the differences between autofluorescence emission of non-neoplastic and neoplastic tissues were much more pronounced in vivo than on excised specimens, indicated the importance of the altered histological organization of tumor, with a greater cell density, affecting the bulk tissue optical properties.

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Reference to a chapter in an edited book:
3. Gmünder FK, Cogoli A. Effect of space flight on lymphocyte function and immunity. In: Fregly MJ, Blatteis CM, eds. Handbook of Physiology. Oxford:University Press, 1996, vol. 2, pp 799-813.

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SCIENTIFIC RESEARCH

The Department of Clinical Physiopathology (DCP) is one of the largest at the University of Florence. Although DCP belongs to the Faculty of Medicine, it is a multidisciplinary department which includes sections with expertises ranging from surgery to endocrinology, from physics to radiotherapy, from genetics to microbiology.

The greatest part of its research activity is supported by the Italian Ministry of Research and by the European Community. Thanks to the scientific outcomes obtained, the DCP has considerable importance within the University of Florence.
Also education has an important role in the activities of the Department. Great care is devoted to the institutional courses of the Faculty. Moreover the DCP organizes many post-doc courses and Masters of the first and second level (it is possible to enter with a three-year or with a specialistic degree respectively). Among these, particularly important is the Master in E-Medicine for training in the field of telemedicine and medical information technology.
The Master on NBC Medicine, dealing with disasters by nuclear, bacteriological or chemical events, is carried out in close collaboration with the Italian Ministry of Defence.

The training on Laser Radiation Safety in Biomedical Environment is addressed to physicians and technical operators, including physicists involved in laser equipment cotrol. It was conceived to inform the learners on the safety standards and how to comply with them. Moreover it is helpful to those who work with light radiation, in order to improve the use of optical instrumentation in total security.

ASAcampus, the ASA Research Division, is housed at the DCP, in the section of Medical Physics. This is the Unit of the DCP that historically studies interaction of light radiation with biological tissues and, more generally, is involved in research on response of cells and biological tissues to physical stimuli. The headquarters of the laboratories is located in a building collecting almost all the Preclinical Research Laboratories of the Faculty, in close contact with researchers of other departments such as Pharmacology, Internal Medicine, Neurological Sciences, etc....This location fosters a continuous exchange of knowledge and skills, allowing an interdisciplinary research with great facilities as regards instrumental systems. ASAcampus laboratories are supplied with advanced facilities for studies on cell biology, biophysics, photobiology, fluorescence and autofluorescence microscopy.

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