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A morphometric and statistical study of the effects of soft laser (He-Ne) irradiation on the pineal gland

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Summary. Thirty-six male Wistar rats (225g average b.w.) were studied after direct He-Ne laser irradiation (632.8 nm, 5 mW) of the surgically-exposed pineal gland. Total irradiation time was 5 minutes, with rest intervals of 1 minute for every minute of irradiation.

The animals were sacrificed in groups of 4 (controls, irradiated animals and sham-operated rats - i.e., craniotomy without irradiation) on days 3, 7 and 10 postirradiation.

A significant increase was seen ($p<0.0001$) in the medullary and cortical karyometric indices of the pineal body in all experimental, control and sham-operated animals. The increase was greatest (over 50%) on day 3 postirradiation among the experimental animals, with respect to the control and sham-operated groups. A progressive and significant decrease in karyometric index ($p<0.05$) was in turn observed on days 7 and 10 postirradiation, although the values remained higher than among the controls.

Ultrastructurally, there were cytoplasmic signs of an increase in metabolic activity in the experimental animals on days 3 and 7, followed by a decrease in activity by day 10 with the appearance of numerous lipid droplets, pericanallicular dark cells and mesoglial cells.

We suggest that laser irradiation stimulates cortical and medullary pinealocytes, followed by a decreased effect at day 10 postirradiation. The effect of laser light is in turn determined by experimental action and the duration of exposure.

Key words: Pineal gland, Karyometric index, Lipid droplets, Pericanallicular cells, Mesoglial cells, Laser irradiation

Introduction

The biological effects of soft-laser irradiation are poorly understood, although most authors have reported important stimulatory effects on both tissue metabolism and bioenergetics (Kolev et al., 1979; Asencio and Martínez-Soriano, 1988; Hernández Gil de Tejada et al., 1990; Silva et al., 1991).

The pineal gland in lower vertebrates is a photo-neuroendocrine organ capable of discriminating between different light wavelengths (Dodt and Jacobson, 1963). In mammals the organ is known to play an important role in endocrine regulation association with variations in the photoperiod through the retinal pathway, (Reiter et al., 1970; Reiter, 1974, 1983; Hoffman and Melvin, 1974) by virtue of its main known hormone, melatonin. The action of this hormone is likewise dependent upon the photoperiod (Sackman et al., 1977; Biswas et al., 1978; Cardinalli and Vacas, 1978; Matt and Stetson, 1979; Bittman and Zucker, 1981).

The physiological variations of the pineal gland with different types of light have also been studied in rodents (Venecek and Illnerova, 1982). Martínez-Soriano et al. (1984, 1990) reported modifications in karyometric index within the pineal parenchyma following 8 minutes of uninterrupted irradiation with coherent light. Similarly, irradiation of the gland with low-power laser light appears to induce functional changes in the gonads of lower vertebrates (Kemali et al., 1981) and in the adrenal glands of mammals (Guillot et al., 1988). Karyometric studies have in turn revealed variations in nuclear size over the 24-hour light cycle. These variations were also established between the cortical and medullary regions of the gland (Cimas et al., 1992). However, such corticomedullary circadian differences were not reported in earlier studies (Welsh et al., 1979; Heidbüchel and Vollrath, 1983). Following previous investigations (Martínez-Soriano et al., 1984, 1990), the reasons for this study and the ones before have been based in part on the work of Slawinska and Slawinski (1983), who affirm the existence of ultraweak cellular radiation that transmits information and regulates fundamental biological functions, and also on the work of Rattemeyer and Popp, (1981); Nagl and Popp, (1983); and Popp and Nagl, (1983) suggesting a correlation between the intensity of photon emission and DNA conformation, with this beings a source of inter and intracellular communication through the transference of photons.

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With these findings, we planned to verify and to study what would occur if a basically photoreceptive organ was irradiated directly with light interfering with its supposed photon code and, in addition, to study what would happen in its target organs.

The purpose of the present paper is to further investigate the corticomedullary karyometric changes and pinealocyte cytoplasmic modifications resulting from soft-laser irradiation of the exposed pineal gland in the Wistar rat; 5-minute irradiation periods with 1-minute rest intervals for every minute of exposure were used. The results obtained were contrasted with those reported in earlier studies.

Materials and methods

Thirty-six male Wistar rats (250±25g b.w.) were divided into three groups of 12 animals each: control; experimental; and sham-operated. All three groups were housed under the same feeding and photoperiodic conditions for the length of the experiment.

Following anaestheia (nembutal i.p., 44 mg/kg), the experimental rats were craniotomized to expose the pineal gland according to the pinealectomy technique described by Sánchez del Campo (1971). The gland was then irradiated with a Polytect 750 He-Ne laser (632,8 nm, 5mW) for a total of five-minutes-applying 1 minute rest intervals for every minute of exposure. The sham-operated animals underwent craniotomy with pineal exposure but not irradiation. The experiments were performed in the second week of November, and between 10:00 and 14:00 GMT.

Following anaesthesia as described above, the rats were sacrificed in groups of four on days 3, 7 and 10 postirradiation, by intracardiac saline perfusion through the left ventricle. This was followed by the perfusion of 5% glutaraldehyde solution in phosphate-buffered saline. Once removed, the pineal gland was fixed and refixed in osmium tetroxide, and then dehydrated in a graded acetone series. The specimens were contrasted with uranyl acetate and embedded in epon.

The 0.5 µm semithin sections of gland for karyometric index evaluation were stained with toluidine blue.

Previous studies (Vollrath, 1981) have shown that 100 nuclei are sufficiently representative, yielding values that differ by no more than ±10% from the mean of 500 measurements. Only clearly visible pinealocyte nuclei were considered.

Volumetric variation was measured for a total of 7200 cells corresponding to all 36 animals; for each rat 20 x 5 medullary and cortical nuclei were measured, i.e., 200 cells per animal. The selection of subsequent sections was made so that each section would be at least 5 µm from the preceding one to thus avoid including more than one section of each nucleus.

The major (A) and minor (B) nuclear diameters were determined under high magnification (x100) using a micrometer fitted to the eyepiece of a Wild-Heerbrugg M20 microscope. Nuclear volume (Vn) was obtained by applying Jacob's formula (1935):
$Vn = \pi/6\ (A.B^2)$ multiplied by a constant, K (microscope magnification).

As it was not the aim of this study to obtain absolute nuclear volumes but to compare different zones and groups of animals, no attempts were made to correct the data and compensate for shrinkage.

For the quantification of lipid droplet areas and pericanallicular and mesoglial cells, sections were cut from each tissue block; the area of the grid with the qualitatively best section was chosen and the tissue overlying 8 apertures (each measuring 65 x 65 µm) was scanned at x12,000 magnification.

The data were processed by a Bull DPS 8/49 computer to compare nuclear volumetric variation in the medullary and cortical zones of the pineal gland in terms of layer, experimental action, and postirradiation time. The BMDP 4V program for mean and standard deviation (SD) analysis was used, along with the SPSS statistical package for variance (ANOVA) and covariance analysis.

Variance, calculated on the basis of the above variables, provides the corresponding dispersion spectrum in terms of the mean of the volumetric mean increases considered for a single variable (experimental action).

Covariance analysis provides similar data, although in this case two variables are considered, i.e., experimental action and postirradiation time.

Results

Karyometric findings

Graphs 1 and 2 show that mean nuclear volume of the pinealocytes in the controls was significantly greater ($p<0.0001$) at medullary level than in the cortex of the pineal gland. This difference persisted throughout the experiment (Table 1).

A significant difference ($p<0.0001$) was likewise seen between the central and peripheral layers in the sham-operated animals, but the mean nuclear volumes corresponding to both layers were still greater than in the controls. These differences were observable by day 3 following the sham operation, reached a maximum on day 7, and then subsequently decreased by day 10. Control values were still not reached, however (Graphs 1, 2).

The changes were more manifest among the irradiated animals; the modifications in mean nuclear volume within the medullary level and cortex were evident by day 3, particularly in the medulla, where the mean volume was over 50% greater than in the controls and sham-operated rats. This difference had progressively decreased by days 7 and 10, but still remained significantly higher ($p<0.05$) than in the other two groups at the end of the experiment (Graph 2).

The differences observed between the sham-operated and irradiated groups persisted in the case of the

Table 1. Variance analysis 1-, 2- and 3-ways interaction.

SOURCE	SQUARED SUM	DEGREE FREEDOM	SQUARED MEAN	VARIAB. F	PROB. p>
Mean	14065608.638	4	3516402.160	313.818	0.
Treatment	07133346.186	1	7133346.186	636.608	0.000
Layer	06580146.161	1	6580146.161	587.238	0.000
Time	00352116.292	2	0176058.146	0.15.712	0.000
2-way interaction	02928477.222	5	0585695.444	052.270	0.
Treatment-layer	02065040.597	1	2065040.597	184.292	0.000
Treatment-time	00794897.610	2	0397448.805	035.470	0.
Layer-time	00068539.015	2	0034269.508	003.058	0.047
3-way interaction	00375138.880	2	0.187569.440	016.739	0.000
Layer-treatment-time	00375138.880	2	0187569.440	016.739	0.000

medullary layer (Graph 2), while the mean cortical nuclear volumes tended to become similar, without significant differences (Graph 1).

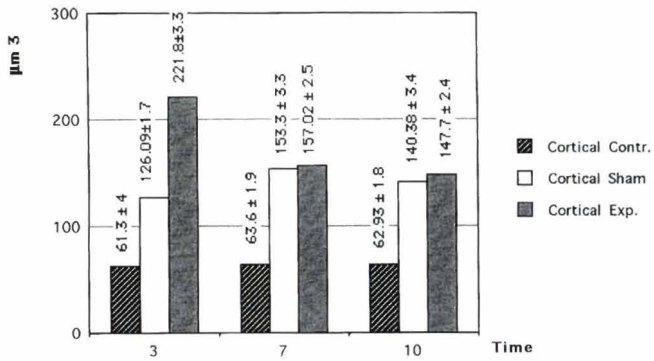
The course of the karyometric indices in both pineal layers was determined by the experimental action applied (irradiation) and by the time of evaluation, the interaction of these two factors being highly significant, as reflected by analysis of variance (ANOVA) (Table 1).

Morphological findings

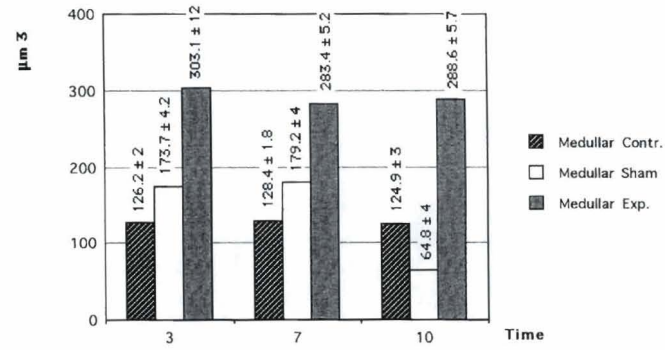
Light and electron microscopy both revealed the presence of a great many lipid droplets (Graph 3), which led us to quantify them in terms of surface area.

Prior sampling showed no significant differences between the medullary and cortical gland region; we thus limited study to the global evaluation of lipids throughout the pineal parenchyma.

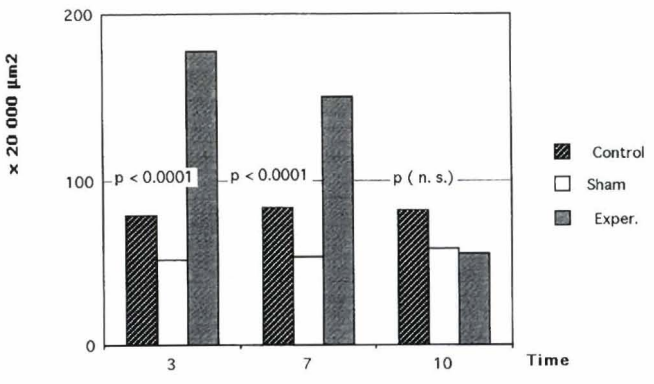
No differences were noted between the controls and sham-operated animals over time. However, on contrasting these results with those of the irradiated group, lipid presence was seen to increase by over 50% at day 3 postirradiation ($p<0.0001$) in the experimental rats. On day 7 this difference had decreased, but was still significant ($p<0.0001$). By day 10 the drop in lipid presence was spectacular (Fig. 1), with levels even lower



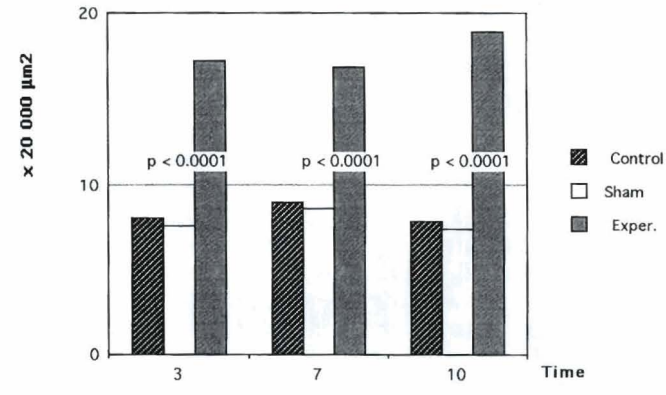
Graph 1. Cortical karyometric index evolution.



Graph 2. Medullar karyometric index evolution.



Graph 3. Lipid «droplets» evolution.



Graph 4. Pericanalicular dark cells evolution.

than in the other two groups (non significant).

In addition to the apparent increase in droplets, a great many perivascular dark cells were observed (Fig. 2).

Ultrastructurally, no significant differences (according to surface measurements) were noted in the number of pericanallicular cells between the medullary and cortical regions in any of the three groups studied. However, as most cells in the controls and sham-operated animals were situated centrally or limiting with the cortical periphery, the region to which effectively they truly belonged was difficult to establish.

The experimental group (Graph 4) presented a very significant increase in these cells compared with the other two groups ($p<0.0001$) on days 3, 7 and 10 postirradiation. Likewise, an increase was noted in the irradiated rats at day 10 in relation to cellularity on days 3 and 7, although this difference was no statistically significant.

Finally, the experimental group exhibited an important number of mesoglial cells on day 10 (8.9/20,000 μm^2) (Fig. 3); this was not observed in the other two groups. These cells were mainly distributed in the more distal peripheral regions supposedly closest to the irradiation one.

Discussion

There is little information in the literature on the effects of coherent light irradiation of the pineal gland. In our study, the karyometric evaluations showed medullary zone nuclear volume to be clearly and significantly greater than at cortical level in the control animals; in agreement with the hypothesis proposed by Quay and Renzoni (1966). This suggests the existence of two functionally distinct regions within the pineal gland of the rat.

The subject of the pineal «cortex» and «medulla» has long been open to controversy. The pioneering studies by Renzoni and Quay (1964) and Quay and Renzoni (1966) revealed variations in nuclear size during the 24-hour cycle, with differences between the peripheral and central regions of the gland. A great many related studies have since appeared in the literature, although a number of authors have questioned the existence of any such cortico-medullary differences (Welsh et al., 1979; Heidbüchel and Vollrath, 1983). Others have supported the presence of differences between the two gland regions (Miline et al., 1969; Blumfield and Tap, 1970), and a number of studies have even reported differences depending on the pineal region studied (Becker and Vollrath, 1983; Diehl et al., 1984), the photoperiod

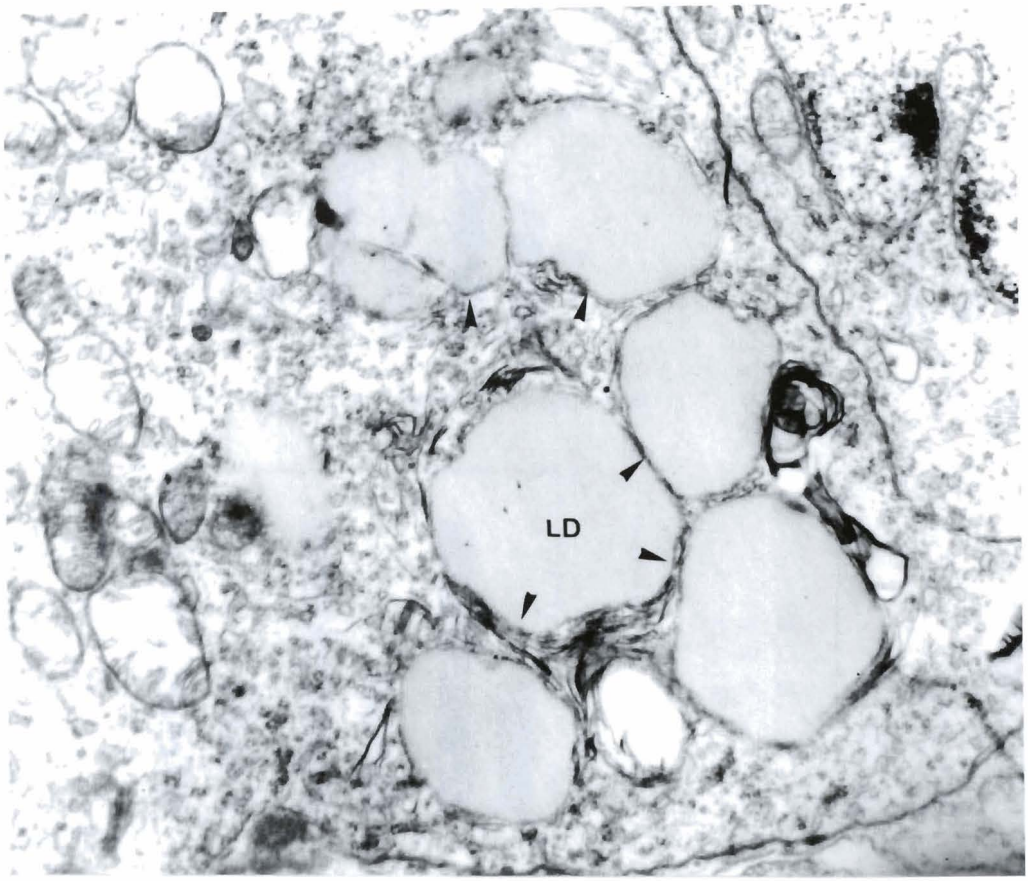


Fig. 1. Detail of experimental group at 10 days after irradiation. See the Lipid Droplets (LD) in the proximity of plasmatic membrane. Lipid droplets are wrapped with a dense peripherical material. Also observe the presence of structured mitochondrias near the LD. x 36,000

(Popova et al., 1975), circadian rhythm (Matsushima et al., 1983), sex of the animal (López-Iglesias and Arias, 1987) or the action of magnetic fields of varying intensity (De La Guardia et al., 1988).

We are of the opinion that each of these factors should be taken into account when evaluating corticomedullary variations in karyometric index; moreover, as suggested by Popova et al. (1975) and Cimas et al. (1988), efforts should be made to establish the determining hour and photoperiod factors when comparing results of this nature.

The results of our study suggest that such corticomedullary differences exist (at least for pinealocyte nuclear volume), as the differences encountered in the control group were again observed among the sham-operated animals, with a marked increase in nuclear volume in both gland regions. These differences were significant 3 and 7 days after experimental action, and tended to decrease by day 10.

These observations suggest the existence of nuclear functional dynamics within the pineal gland, in turn clearly influenced by the simulated operation (craniotomy without irradiation).

Environmental light may be thought to account for these modifications; however light is considered to inhibit pineal function. Accordingly, nuclear volume should decrease rather than increase with exposure, although Vollrath (1981) has reported elevated gland activity and hence increased nuclear volume after prolonged periods of continuous illumination. One of the determining causes of the increase in pinealocyte nuclear volume in our study may be surgical stress, particularly when taking into account that volume increase was greatest over the first 7 postoperative days, followed by a steady decrease.

Among the irradiated animals these variations were accentuated; thus nuclear volume had clearly increased in both pineal layers by day 3 postirradiation, followed by a progressive decrease until the end of the experiment at day 10. This decrease was greater and more rapid in the cortical region than in the medulla, where although a slight initial increase in volume was noted, the situation remained practically stable between days 7 and 10 postirradiation.

Our results thus agree with the hypothesis proposed by Quay and Renzoni (1966) and corroborated by other authors (Romijn, 1973; Popova et al., 1975; Cimas et al., 1988; De La Guardia et al., 1988; Giménez et al., 1991); i.e., nuclear activity is greater in the medullary region of the pineal gland than at cortical level, at least between 10:00 and 14:00 (GMT) during the winter season.

The morphological results obtained in the present

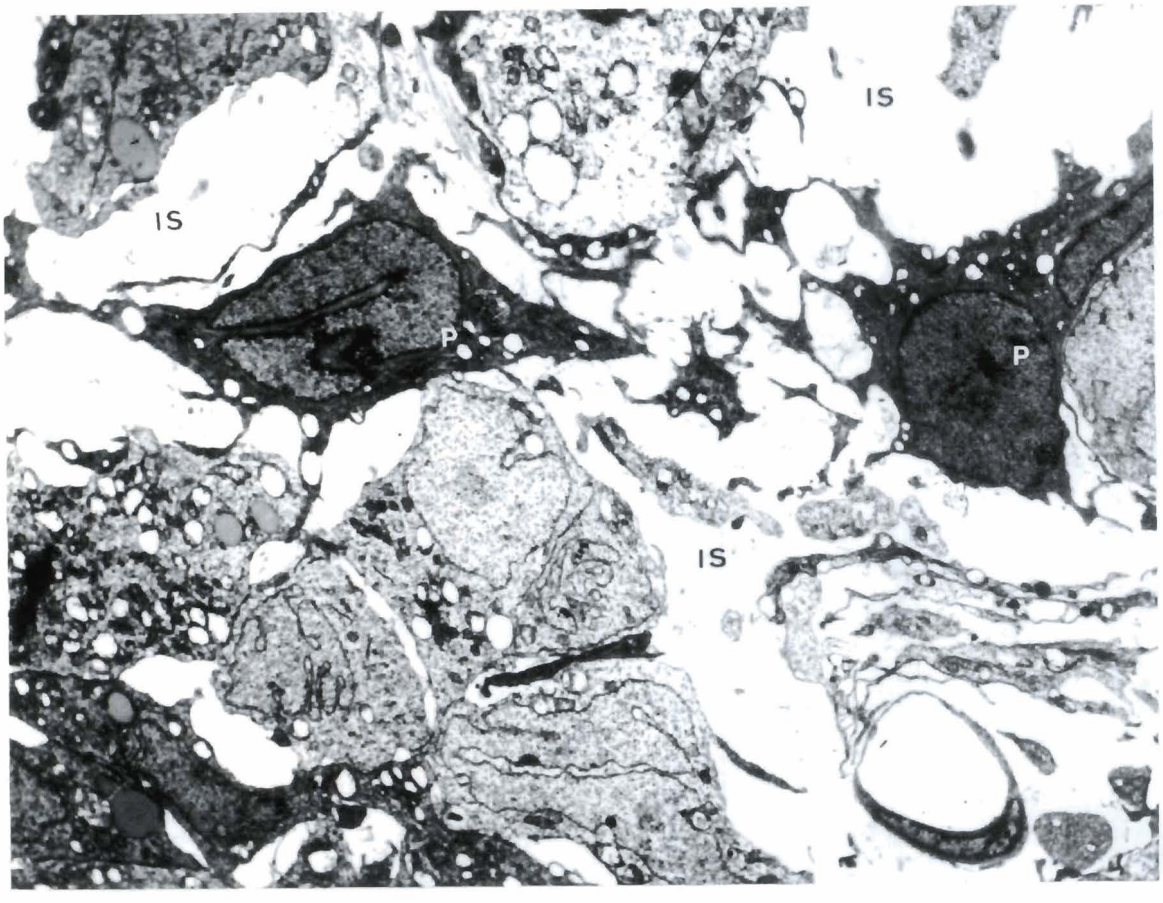


Fig. 2. Perivascular dark cells (P), Type II pinealocytes with typical dark cytoplasm in the experimental group 10 days after irradiation. See the intercellular spaces (IS) containing vesicles and granular material. x 12,000

study reflect an important change in the presence of lipid droplets within the pineal gland following laser irradiation. A number of authors have found lipid droplet variation and size to directly depend upon pinealocyte metabolism and its circadian changes (Quay, 1957; Zweens, 1963, 1965; Miline et al., 1969). From the start, these droplets were thought to be pineal secretion products. Quay (1974) considered them to play a fundamental role in general and primary pinealocyte metabolism, and reported them to be more abundant within the medullary region of the gland than at cortical level. On the contrary, López Iglesias and Arias (1987) found droplets to be more abundant in the cortical region in female rats.

Our results failed to show significant differences between the medullary and cortical zones in any of the group studied. On evaluating droplet presence globally, highly significant increases ($p<0.0001$) were observed in the irradiated group by days 3 and 7 in relation to the controls and especially the sham-operated rats. The actual decrease observed in the sham-operated group could be explained in terms of the observations reported by Quay (1974), Zboray (1965) and Prop and Ebels (1968), who found that light reduces pinealocyte lipid content. In effect, the pineal gland in the sham-operated animals was exposed to environmental light during craniotomy.

The great increase in lipid droplets observed between days 3 and 7 postirradiation could be the result of an initial stimulating effect of the laser light, followed by a sudden drop in function by day 10.

Physical factors such as immobilization (Miline et al., 1969) or exposure to pulsating magnetic fields (Giménez et al., 1991) also cause prompt significant increases in lipid droplet content within the pineal body.

Martínez Soriano et al. (1990) have reported spectacular non-quantified increases in lipid droplets following laser irradiation of the pineal gland in the rat; however, this increase was accompanied by a simultaneous decrease in nuclear volume from the first postirradiation day onwards.

The interaction of laser light with living tissue apparently exerts different effects, depending on the energy density employed. Mester (1982) reported that low-power coherent light has a biostimulating effect, the latter being accumulative for successive doses until an inhibitory effect may finally result. According to Brown (1983), all doses delivered at less than 4 J/cm^2 have a

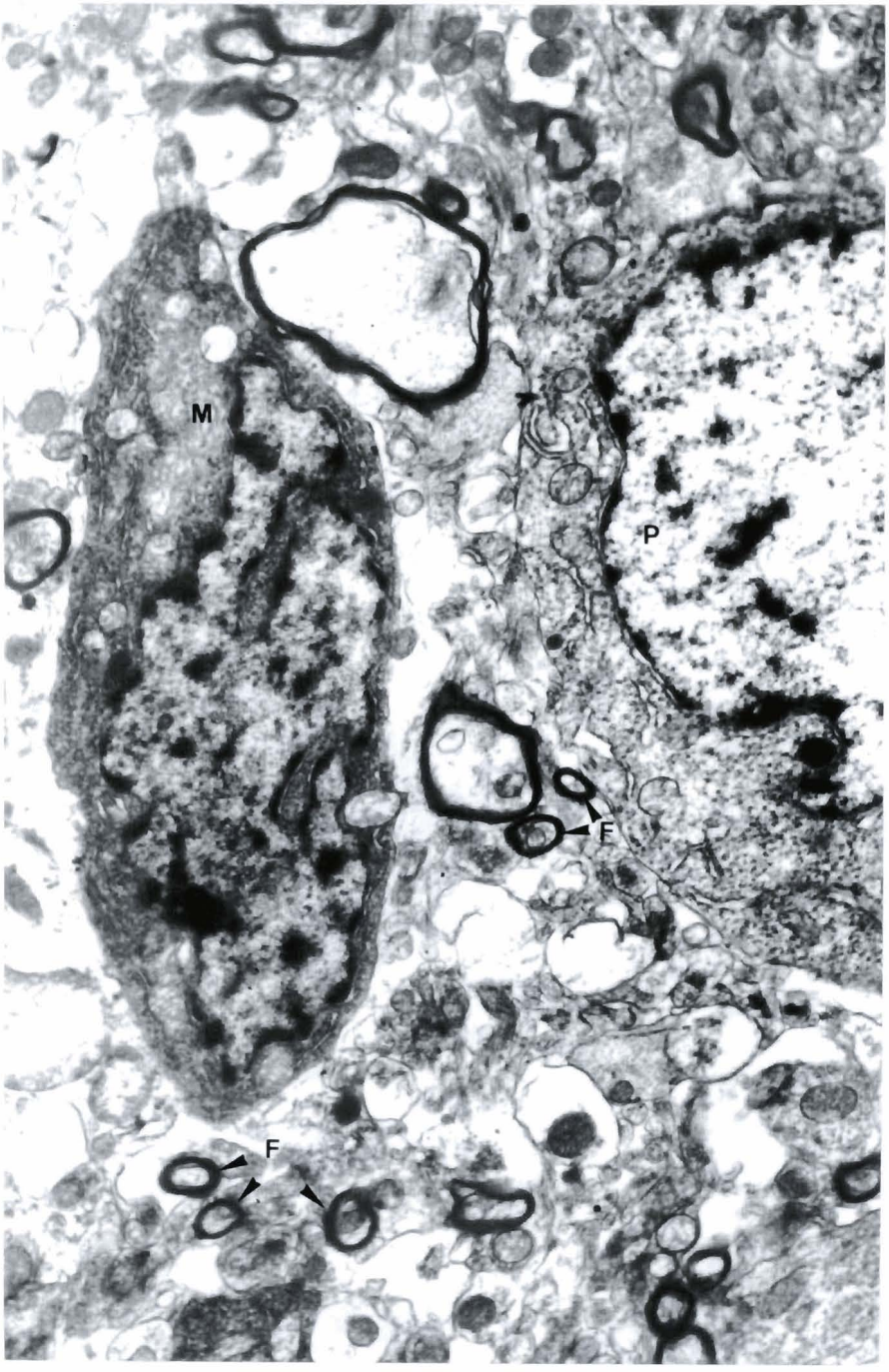


Fig. 3. Mesoglial cell (M) near a pinealocyte (P) surrounded by myelinic fibres (F) and protoplasmic prolongations of pinealocytes. Experimental group at 10 days. x 24,000

stimulating effect, whereas higher energy densities are inhibitory.

In the present study we employed laser doses corresponding to 1.17 J/cm^2, i.e., irradiation was supposedly within the biostimulatory limits. This was reflected by our findings on days 3 and 7. However, by day 10 this stimulatory effect had largely disappeared - possibly due to functional depletion following the initial stimulatory phase. In agreement with Mester (1982), irradiation sessions interspaced by rest intervals might lead to an accumulation of energy, and hence to a long-term inhibitory effect. In turn, it may be suggested that the seasonal photoperiod induces a different gland response based on the essential photoreceptive nature of the latter.

Another interesting finding in the present study was the progressive increase in pericanallicular dark cells after day 3 postirradiation. The literature (Vollrath, 1981; Martínez-Soriano, 1987) does not clarify whether these cells represent a different functional stage of the type I cell in Pevet's classification (1977), or whether they correspond to protoplasmic astrocytic glial cells. We are inclined to agree with Schachner et al. (1984), who suggest that these cells are really immature astrocytes. The observations by Martínez-Soriano et al. (1990) support this hypothesis. They found that pericanallicular cells in rats subjected to laser irradiation develop a rich fibrillar cytoplasmic network similar to that encountered in the astrocytic glia at the gland periphery. This suggests that the pericanallicular cells are indeed immature astrocytes, and that coherent light causes them to activate and multiply, as seen in our study (Graph 4).

Finally, the signs of diminished nuclear and cytoplasmic activity observed by day 10 postirradiation in the experimental animals, together with the appearance of a microglial cell population suggest that following an initial activation phase, laser light at the doses utilized exerts a lesive effect. Martínez-Soriano (1990) likewise observed a great mesoglial invasion of the pineal gland 15 days after a continuous 8-minute irradiation session.

The pineal gland is known to exert a regulatory effect on endocrine function, an effect in turn fundamented on the length of the photoperiod. Light generally reduces pineal function, and consequently activates the endocrine system. Laser light at the doses employed in our study appears to have an initial stimulating effect, followed by inhibition, the latter possibly depending on the accumulative effects of irradiation. However, the relatively prompt appearance of microglia after the initial active phase suggests that irradiation following our experimental protocol causes important damage.

In this sense, unpublished observations in the same groups of rats show the appearance of cysts within the fascicular layer of the adrenal glands, with morphological signs of liver and gonadal activation followed by organ hypofunction.

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