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Static electromagnetic field effects on pineal vesicles: A morphological and morphometric study in chick embryos

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SUMMARY

The effects of static electromagnetic field exposure on the development of pineal cell aggregates were studied in chick embryos. Static electromagnetic fields were created by a solenoidal coil (110x160 mm) kept inside the incubators and connected to a power supply. The magnetic field generated was static and uniform (frequency = 0; wavelength = 0) and the intensities were 18 mT and 36 mT. Eggs were incubated either under control or static electromagnetic field conditions, and embryos were sacrificed after 5, 10 and 15 days of incubation. The morphology, number and density of pineal aggregates were determined in three pineal areas (apical, anterior and posterior). In exposed embryos, the vesicles appeared earlier (by the 5th day) and after 15 days of incubation the number of vesicles was higher and stroma compaction was greater than in controls. These changes in vesicle morphology and density suggest that a static electromagnetic field might be able to stimulate pineal maturation and development in the chick embryo.

Key Words: Development - Embryology - Chicken - Epiphysis

INTRODUCTION

Interest in the effects that exposure to electromagnetic fields may have on health has been increasing in recent years (Savitz et al., 1989; Reiter, 1994, 1995). The latter authors agree that nerve and bone tissue, and blood cells, particularly if they are developing, are affected by electromagnetic fields, (Abdullakhdzhayeza and Razykov, 1986; Espinar et al., 1997; Piera et al., 1997). Some authors (Adey, 1993; Savitz et al., 1989; Chernoff et al., 1992; Walleczek, 1992; Frey, 1993; Shaw and Croen, 1993; Stevens, 1993; Hughes, 1994; Hendee and Boteler, 1994) believe that there is a relationship between the functional state of the pineal gland and the appearance, or risk of appearance, of certain pathological processes (cancer, reproductive disorders, depressive states, etc.). Likewise, the relationship between electromagnetic fields and the pineal gland has been well documented ever since it was shown that one could modify the other, (Wilson et al., 1989; Olcese, 1990; Semm et al., 1980; Reiter, 1994). A correlation between the pineal gland, the central nervous system, immune system and electromagnetic fields has also been reported (Frey, 1993; Janckovic et al., 1994).

Pineal vesicles are responsible for storing the products of pinealocyte secretion (Greve et al., 1993; Nowak et al., 1997) and modifications to the vesicular tissue may lead to modifications in the secretion of pineal products. In 1999 Jové et al. studied the evolution and distribution of pineal gland cellular aggregates in chick embryos at different stages of development.

Interest was aroused in the morphology and distribution of pineal vesicles when it was confirmed that in chicks melatonin synthesis begins

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during the embryonic period (Wainwright, 1974; Greve et al., 1993; Akasaka et al., 1995). Although the histological, immunohistochemical and physiological features of the pineal glands of vertebrates (not birds) have been carefully investigated, studies on chick embryos are not so numerous (Quay, 1965; Ralph et al., 1975; Doskocil, 1976; Omura, 1977; Calvo and Boya, 1978; Binkley, 1979, 1980; Goto et al., 1989, 1990; Möller and Möller, 1990; Jové et al., 1999a). Likewise, histological studies on pineal vesicles are mainly based on qualitative observations while fewer quantitative data have been reported (Spiroff, 1958; Campbell and Gibson, 1970; Jové et al., 1999a). Electromagnetic fields modify the function of the pineal gland, as evidenced by changes in melatonin secretion (Reiter, 1994).

In this study our aim was to determine whether electromagnetic fields are able to change the morphology and distribution of cellular aggregates in the pineal gland of developing chicks and whether such changes are indicative of the functional activity of the gland.

Materials and Methods

Static electromagnetic field (SEMF) Exposure System And Incubation Conditions

Two identical incubators (Masalles, model 25, Spain) equipped with a temperature selector, thermostat, potentiometer, hygrometer and automatic egg turnover device were used. The static magnetic field exposure system used has been described previously (Piera et al., 1992; Espinar et al., 1997). Briefly, the SEMF was created by a solenoidal coil, consisting of a rigid PVC support and a monofilament, kept inside each incubator. The center of the coil was a hollow cylinder 110 mm in diameter and 160 mm in length. The solenoid was connected to a power supply (Promax FAC-5528, Spain) located outside the incubators and a SEMF was obtained (frequency = 0; wavelength = 0). The intensity of SEMF could be controlled between 0 and 40 mT.

One incubator was always used with the power source disconnected from the coil. The eggs incubated in this way were the control groups. The second incubator was always used with the coil connected to the power source. The eggs incubated under these conditions were considered to be those exposed to a SEMF. Some batches were incubated under the effect of a SEMF of 18 mT while others were subjected to 36 mT.

All incubation conditions were controlled before, during and after each experiment. The eggs were incubated in darkness and turned automatically once every hour. Temperature (37.5 (0.1°C) was checked with a sensor located in the center of the egg support. The hygrometer, located inside the incubator, showed humidity to be above 70%all the time. The static electromagnetic fields measured with a gaussmeter (Oxford Instruments, model kGauss-031T, Spain) in the laboratory room and inside the incubator with the static electromagnetic field system turned off was approximately 0.044 mT. All these parameters were maintained constant during the study.

Eggs

Fresh fertile White Leghorn eggs were purchased from a commercial hatchery (Nueva Comarcal, Reus, Spain). Before incubation, the eggs were stored horizontally for 24 h at 4°C with the small end of the egg facing the magnetic south of the earth. During the experiment, the eggs were placed horizontally in a specially designed amagnetic support which was then introduced into the centre of the coil in such a way that their pointed end faced the magnetic south of the static electromagnetic fields generated.

A total of 144 fertile White Leghorn eggs were used, 48 of which were incubated under control conditions (incubator with the coil disconnected), and 96 of which were incubated under the effects of the SEMF (incubator with the coil connected). Of the latter, 48 were submitted to an SEMF of 18 mT and 48 to an SEMF of 36 mT. A third of the embryos in both the control and the exposed groups were sacrificed after 5 days, another third after 10 days, and the final third after 15 days of development. The embryos of the exposed groups were subjected to the SEMF continuously, from day 0 of incubation to the moment of sacrifice.

The eggs from the same lay were randomly assigned to a dose group and their day of sacrifice was randomly assigned by computer. The weight, the height and the Hamburger and Hamilton (HH) stage were determined in each of the embryos, using the Hamburger and Hamilton method (1951).The groups were not identified until the end of the statistical study.

Light Microscopy and Morphometric Study

The conventional method to process the embryos was used. The embryos were fixed in 10% formaldehyde buffered solution and then embedded in paraffin. Seven-micrometer serial sections (in the sagittal or frontal planes after 5 and 10 days and sagittal and transversal planes after 15 days) were made and H-E staining was applied. The 15-day-old embryos were decalcified by chloral hydrate.

The sections were observed using a binocular stereoscopic magnifier (Zeiss) equipped with a micrometer ruler. Vesicle diameters and densi-

ties were determined using a semiautomatic image analysis system (Mop-Videoplan 2000, Kontron). These data were obtained from the image projected on a television monitor (Sony Trinitron RGB, KX-14CP1), using an optical reading pencil.

Section Selection

Sagittal sections (Fig. 1, S): After we had observed all the sagittal sections of each of the embryos after 5,10,and 15 days of development, we selected one section per embryo on which to carry out all the determinations. The sagittal section selected was the middle one: that is to say, of all the sections in which the pineal gland could be seen, we chose the one which had the same number of sections forward and rear to it. To standardise the observations on the selected sections, we used a coordinate system. The horizontal axis was a line tangential to the diencephalic roof drawn from the angle that the roof forms with the pineal gland. The vertical axis was a line perpendicular to the horizontal axis and tangential to the posterior-most end of the gland. These axes enabled us to delimit three areas:

1. The apical area (APL) was the region delimited by the distal surface of the gland and the line parallel to the vertical axis and tangential to the distal-most end of its lumen.

2. The anterior area (AA) was the region delimited by the anterior wall of the gland, its lumen, the portion of the horizontal axis which is the base and the limit of the apical region.

3. The posterior area (PA) was:

- After 5 days, the region delimited by the posterior wall, its lumen, the portion of the vertical axis which is the base, and the limit of the apical region.
- After 10 and 15 days, the region delimited by the posterior wall, its lumen, the portion of the horizontal axis which is the base, and the limit of the apical region.

Frontal sections: After we had observed the whole series of frontal sections of the embryos after 5 and 10 days, two sections were selected for each embryo so that the three areas defined could be observed. Section F1 was the third section after the observation of the lumen from the anterior end (Fig. 1, F1), and it showed the anterior and posterior areas. Section F2 was the one before the appearance of the glandular lumen (Fig.1, F2); the whole of the section shows the apical area of the gland.

The axes defined were: The vertical axis was the shortest distance between the gland and the diencephalon roof. Two horizontal axes, perpendicular to the vertical axis, were located tangential to the superior and inferior end of the lumen. The anterior area was the region delimited by the anterior wall and the inferior axis; the posterior wall of the gland and the superior axis delimited the posterior area.

Transverse sections: After we had observed the whole series of transverse sections of the embryos after 15 days of development, two sections were selected for each embryo so that the three areas defined could be observed. T1 was the third section after the glandular lumen from the pineal cranial end (Fig. 1, T1); the apical and posterior areas can be seen. T2 was the third section after the appearance of glandular lumen from the pineal caudal end (Fig. 1, T2) and it showed the anterior and posterior areas. In both sections, the horizontal axis of the coordinates defined was the line joined the two ends of both cerebral hemispheres and the vertical axis was a line perpendicular to the anterior line which cuts the glandular lumen at the midpoint of its amplitude. The apical and anterior areas were the regions delimited between the glandular walls and an axis parallel to the horizontal one and tangential to the anterior end of the glandular lumen in both sections T1 or T2, respectively. The posterior area was the region delimited by the glandular walls and a line parallel to the horizontal axis and tangential to the posterior end of the lumen in both sections (Fig. 1, T1 and T2).

Pineal Cell Aggregates

After the previous study, the following cellular groups were observed in all the sections and in all the embryos:

- Rosettes: groups of cells arranged radially with a diffuse central zone and showing no sign of a central lumen.
- Vesicles: groups of cells arranged radially, with a well defined central lumen.

For each of the sections selected from all the embryos, the glandular area was determined and the rosettes and/or vesicles were counted. From the relationship between the total number of cellular aggregates and the glandular area, the density per mm^2 was determined.

Twelve ten-day embryos (4controls, 4 exposed to 18 mT and 4 exposed to 36 mT) and twelve fifteen-day embryos were chose from among all the sagittally-sectioned embryos and their morphology and vesicle size were studied. Nine vesicles were chosen in each embryo (3 per area). All the vesicles in all the sections of the embryos selected were studied and the greatest and smallest diameter of each of the vesicles was determined. Two types of vesicles were defined:

- Round vesicles: those in which the ratio between the greatest and smallest diameter was below 1.5.
- Ellipsoidal vesicles: when the ratio between both diameters was greater than or equal to 1.5.

Subsequently, in all the ten- and fifteen-day-old embryos the number of round and ellipsoidal vesicles was determined per surface unit (density) in each of the areas determined.

Statistical Analyse

All the parameters were measured in a blind trial by a single observer who was unaware of the group to which the embryos belonged. Results are expressed as means and standard deviations. The Kruskal-Wallis test was carried out to detect statistical differences among the three groups (controls, embryos exposed to 18 mT and those exposed to 36 mT). When the Kruskal-Wallis test was significant ($p<0.05$), the non-parametric U-Mann-Whitney test was carried out to compare the control group and each of the exposed groups. The χ^2-test was implemented to compare the HH-stage among the three groups.

Results

Weight, Height and HH Stage

On the 5th day, there were no differences in height and weight between the various embryo groups. However, on the 10th and 15th day, the height and weight of the exposed embryos were greater than those of the controls ($p<0.01$, Table 1).

After 5 days, the HH stage of all embryos from the control group and the group exposed to 36 mT were between 24 and 26, while the stages of 26% of the embryos from the group exposed to 18 mT were higher than 27 ($p<0.01$).

In the ten-day exposed embryo group, a statistically high percentage (50%) of embryos was found to have a more advanced HH-stage than the controls ($p<0.01$). Likewise, in the 15-day exposed embryo group, a statistically high percentage (25%) was found to have a more advanced HH-stage than the controls ($p<0.01$).

Density of Cellular Aggregates

After five days, rosettes were the cellular aggregates that were found in the highest percentages. They were present in all the areas of the three groups of embryos. In both groups of exposed embryos, the percentages of cellular

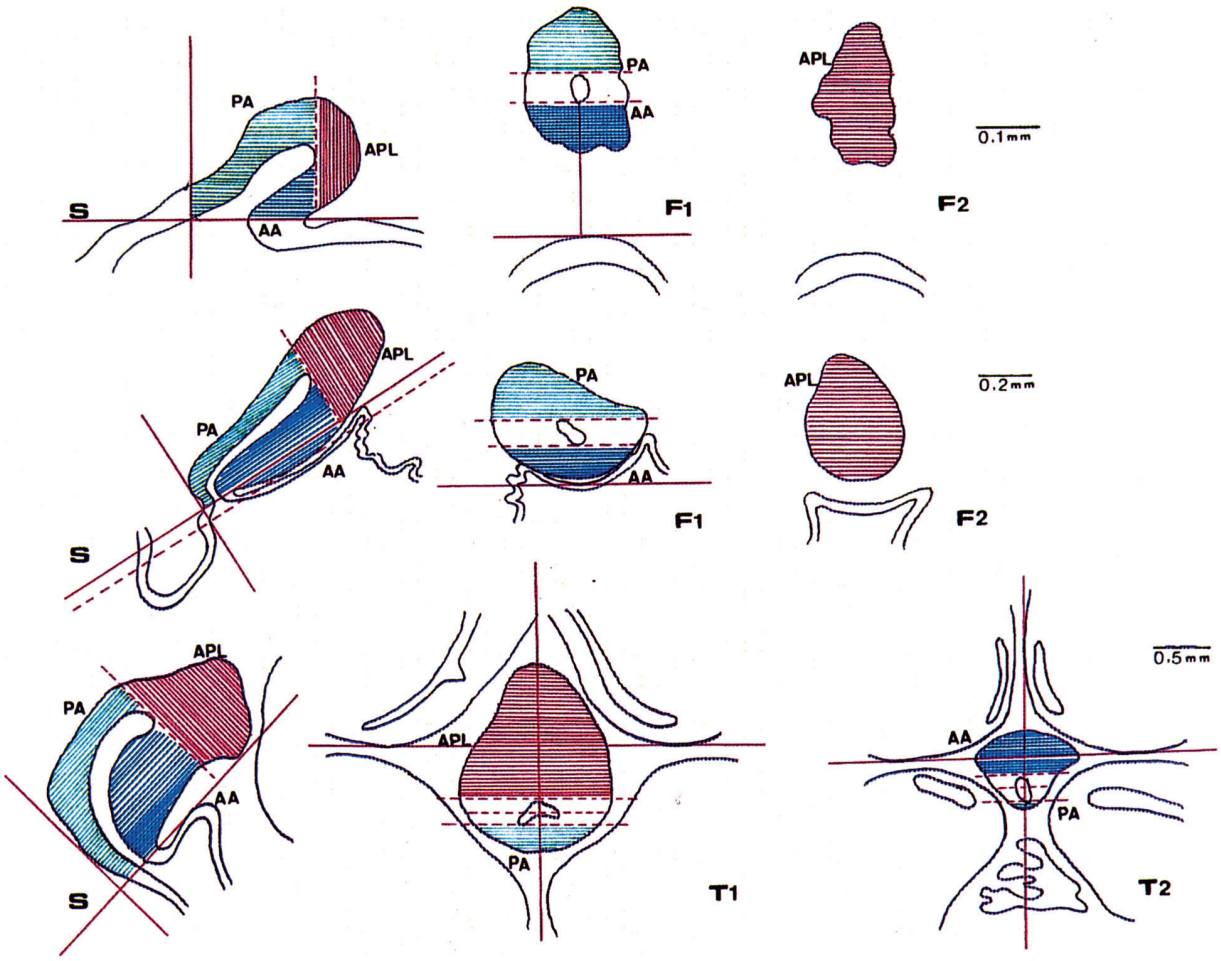

Fig. 1.- Pineal gland drawings after 5 (upper), 10 (middle) and 15 (lower) days of incubation.
APL = Apical Area. AA = Anterior Area. PA = Posterior Area. S = Sagittal section.
F1 = third frontal section after the appearance of the glandular lumen.
F2 = first frontal section before the appearance of the glandular lumen.
T1 = third transverse section after the appearance of the glandular lumen from the pineal cranial end.
T2 = third transverse section after the appearance of the glandular lumen from the pineal caudal end.

aggregates were higher than those of the controls in all areas. Likewise, vesicles were found in all the areas studied of the embryos from the exposed groups. In the controls, however, only one vesicle was found in the apical area of one of the embryos. The differences detected in the density of the cellular aggregates from the embryos exposed to a continuous SEMF of 36 mT and the controls were statistically significant ($p<0.01$, Table 2).

On the 10th day, in SEMF-treated embryos, the vesicles were the only cellular aggregates that could be seen in each area and each embryo group. Cellular rosettes were only detected in the control embryo groups (Table 2). Furthermore, the vesicular density of embryos exposed to 36 mT SEMF was lower than in controls ($p<0.01$), (the opposite of the situation on the 5th day). The glandular surface in this exposed group was greater than in the controls (Table 2), but the number of vesicles in the 3 areas of both the exposed and the control groups was exactly the same.

After 15 days, there was a considerable increase in the number of vesicles in the three groups of embryos and no rosettes were observed in any of the groups studied. The density of the aggregates was greater in both exposed groups than in the controls while the glandular surface was smaller in both exposed groups than in the controls. The differences detected in these two variables between the embryos exposed to 36 mT and the controls were statistically significant ($p<0.001$, Table 2).

Shape and Size of the Vesicles

The study was carried out in the various areas in the pineal gland. The statistical tests showed that the anterior and apical areas had no differences in morphology or vesicle size. Therefore, they were studied together. Because of the differences found between the vesicular sizes and shapes, the posterior area was studied separately (Table 3).

In the posterior area of all the groups of embryos after both 10 and 15 days, all the vesicles observed were round and smaller than those in the anterior and apical areas.

In the anterior and apical areas of all three groups of embryos, after 10 days of development there were more round vesicles than ellipsoidal ones and they all had diameters that were smaller than in the controls.

Also in the anterior and apical areas, after 15 days, there were considerably more ellipsoidal vesicles in the two groups of exposed embryos. The vesicular diameters were smaller in the embryos exposed to 36 mT than in the controls, and this proved to be statistically significant ($p< 0.05$).

Vesicular Density

The vesicular density was calculated in the three areas of all the sections selected from the three groups of embryos after 10 and 15 days (Table 4).

The only differences in density which proved to be statistically significant were found in the 15-day-old embryos ($p<0.01$). Thus, the embryos exposed to a SEMF of 36 mT had a greater vesicular density than the controls in the three areas studied and of both types of vesicles, with the only exception of the ellipsoidal vesicles in the posterior area. This was also the case for the embryos exposed to a SEMF of 18 mT: all the density values were higher than those of the controls. However, these differences only proved to be statistically significant in the anterior area.

Discussion

The presence of vesicles in the pineal gland of the chick embryo has been described by Spiroff (1958), Campbell and Gibson (1970), Calvo and Boya (1978), Ohshima and Matsuo (1988), and Jové et al. (1999a). According to Calvo and Boya (1978), the pineal gland grows because of the proliferation of cells which form rosettes and which subsequently change into vesicles when the central lumen appears. In subsequent studies, Jové et al. (1999a) confirmed these results and described two different vesicular shapes: spherical and ellipsoidal. They suggested that the different morphologies may correspond to different developmental stages, which in turn may be related to the secretory activity of the gland.

In this study, on the 5th day of incubation only cellular rosettes were observed in the pineal gland of control embryo groups while some vesicles could be seen in the pineal gland of the exposed embryos. This seems to confirm that SEMF affect developing organisms. Authors such as Duriez and Basset (1980) and Zhang and Whittow (1993) have shown that SEMF can accelerate the processes of differentiation and/or growth of several tissues in different animal species. This is confirmed by the data obtained here and elsewhere (Piera et al., 1992, 1997, 1999; Jové et al., 1999b) which show that, unlike embryos exposed to a pulsating magnetic field of low and high frequency, embryos exposed to a continuous magnetic field are heavier, larger and have a more advanced HH stage (Berman et al., 1990; Chacon et al., 1990; Yip et al., 1994).

At later stages (on the 10th and 15th days), we observed that the only type of cellular aggregate present in the pineal gland was the vesicle (except on the 10th day, when some cellular

Table 1.- Weight and height of embryos.

Days	Group	n	Weight (g)	Height (mm)
5	control	16	0.20±0.08	12±5
	18 mT	16	0.23±0.07	11±2
	36 mT	16	0.16±0.06	10±2
10	control	16	2.2±0.4	27±4
	18 mT	16	2.8±1.0	32±3*
	36 mT	16	2.4±0.7	32±9*
15	control	16	11±0.7	49±6
	18 mT	16	15±1.5*	57±9*
	36 mT	16	14±1.3*	59±10*

*p < 0.01 vs controls.n = number of embryos

Table 2.- Glandular surface, number and density of cell aggregates.

Days	Group	n	Glandular surface mm²	Cellular aggregates number	Density number/mm²	Rosettes %	Vesicles %
5	Control	16	0.028±0.007	6±3	285±214	100	--
	18 mT	16	0.025±0.010	7±5	310±258	92±12	100
	36 mT	16	0.019±0.005	7±5	421±368*	86±13	100
10	Control	16	0.33±0.08	17±5	52±51	12±8	88±17
	18 mT	16	0.33±0.09	16±3	48±48	0	87±16
	36 mT	16	0.44±0.09	16±4	36±36*	0	87±18
15	Control	16	0.84±0.20	15±2	18±14	0	68±24
	18 mT	16	0.82±0.37	19±10	26±17	0	68±18
	36 mT	16	0.69±0.21*	29±14*	49±15*	0	61±14

*p < 0.01 vs controls. n = number of embryos

Table 3.- Major and minor diameters (mm) of vesicles in control and exposed embryos.

		APICAL + ANTERIOR AREA						POSTERIOR AREA		
		Sphere-like vesicles			Ellipsoid vesicles			Sphere-like vesicles		
Days	Group	V	MØ	mØ	V	MØ	mØ	V	MØ	mØ
10	Control	16	0.13±0.02	0.12±0.03	8	0.12±0.04	0.05±0.01	12	0.06±0.02	0.05±0.02
	18 mT	19	0.12±0.03	0.10±0.04	5	0.09±0.03	0.06±0.02	12	0.07±0.01	0.06±0.01
	36 mT	20	0.11±0.04	0.09±0.03	4	0.07±0.01	0.05±0.02	12	0.07±0.01	0.06±0.01
15	Control	15	0.16±0.03	0.15±0.01	9	0.28±0.04	0.16±0.03	12	0.10±0.08	0.09±0.07
	18 mT	7	0.13±0.02	0.13±0.02	17	0.27±0.05	0.10±0.06	12	0.08±0.04	0.07±0.04
	36 mT	8	0.11±0.04	0.11±0.03*	16	0.24±0.05	0.09±0.01*	12	0.10±0.06	0.09±0.06

*p<0.05 vs controls. V= number of vesicles. MØ = major diameter mØ = minor diameter

Table 4.- Density (number/mm²) of pineal vesicles.

Days	Group	n	APICAL AREA		ANTERIOR AREA		POSTERIOR AREA	
			Sphere-like	Ellipsoid	Sphere-like	Ellipsoid	Sphere-like	Ellipsoid
10	Control	16	40±22	8±9	63±29	9±6	66±57	0
	18 mT	16	44±16	6±9	53±27	6±10	50±31	0
	36 mT	16	46±17	5±7	44±32	7±10	53±37	0
15	Control	16	20±9	8±5	14±8	7±10	17±22	3±17
	18 mT	16	22±8	11±6	36±22*	16±11*	20±16	3±11
	36 mT	16	39±8*	19±13*	38±37*	17±16*	34±26*	5±18

*p<0.01 vs controls. n = number of embryos

rosettes were found in the control group). The differences detected between the 15-day embryos exposed to a SEMF of 36 mT and the controls seem to indicate that exposed embryos are more mature.

In a study carried out on animal species other than the chicken, Sakai et al. (1996) detected differences in the development of the pineal gland and suggested that they were directly related to a different functional activity.

Calvo and Boya (1978) showed that the pineal gland of the chicken embryo develops principally at the expense of the anterior and apical areas. Jové et al. (1999a) confirmed this and stated that the morphology, size, and number of the cellular aggregates depend on the area of the gland in which they are found (anterior, posterior or apical).

According to Campbel and Gibson (1970), the gland increases most in size between days 7 and 10 of embryo development. Our study seems to confirm this since the lower density detected in the 10-day-old embryos exposed to a SEMF of 36 mT is not due to an increase in the number of vesicles but to an increase in the glandular surface.

Several authors have shown that the pineal gland grows rapidly until the twelfth day of incubation. After this time, there are only small changes in volume until birth (Spiroff, 1958; Calvo and Boya 1978; Jové et al., 1999a). Nevertheless, in this period the pineal parenchyma continues to grow and this leads to a gradual decrease in the amount of stroma, giving the pineal gland a compact appearance.

In the present study, the main differences between the exposed (particularly those exposed to 36 mT) and control embryos were observed on the 15th day, which is when the period of gland activity and the compaction process begin. In the control group, between the 10th and the 15th day the number of vesicles remained practically unchanged, whereas in the group exposed to 36 mT the number and density of vesicles increased significantly.

The smaller size of the vesicles, together with the increase in their number and the decrease in the glandular surface, may point to a greater degree of differentiation of the gland. This result is consistent with that of the morphometric study carried out by Jové et al. (1999b) on the pineal gland of the chicken embryo, showing that the glandular diameters of 15-day-old embryos exposed to SEMF are smaller than those of controls.

Calvo et al. (1997) have suggested that diurnal and nocturnal variations in the volume of the pineal are due to changes in melatonin secretion. Likewise, the reduction in the cytoplasmic mass of the pineal cells, and consequently in the size of the pineal gland, has been identified as an index of the decreasing secretory activity of pineal cells (Quay 1965; Ito and Matsushima 1967; Diehl et al., 1984; Matsushima et al., 1990).

Many authors have observed that the pineal gland seems to be sensitive to SEMF (Semm et al., 1980; Grota et al., 1994; Reiter, 1994; Selmaoui and Touitou, 1995) and that this sensitivity is reflected in a decrease in melatonin synthesis. Greve et al. (1993), Akasaka et al. (1995) and Nowak et al. (1997) have described the mechanisms that regulate melatonin synthesis and explained the presence of secretion products in the vesicles. Several histochemical studies have shown that the main secretory function of the pineal gland of the chicken embryo takes place between the 10th and the 17th day of incubation and that after the 17th day this activity decreases (Campbell and Gibson 1970; Greve et al., 1993).

In the light of these results, it may be assumed that the greater compaction of the gland in the 15-day embryos exposed to SEMF (particularly in those exposed to 36 mT) reflects an acceleration of the maturation processes. This may be related to possible modifications in the functional activity of the gland. However, further studies should be designed and carried out if this is to be confirmed.

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