Rat pinealectomy. A modified direct visual approach[1]

Pinealectomia em ratos. Técnica modificada com visualização direta

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ABSTRACT

Purpose: To report a new, direct visual approach for rat pinealectomy. **Methods**: Eighty adult female rats (*Rattus norvegicus albinus* EPM-1 strain) were weighted and anesthetized intraperitoneally with 15 mg/kg xylazine and 30 mg/kg ketamine. The animal was fastened to a dissection table, an incision was made in the skin and the subcutaneous tissue, bringing the lambda into view. The skullcap was opened with a dental drill, bringing the cerebral hemispheres and the superior sagittal sinus into view. The pineal gland, located under the venous sinus, was removed in a single piece using tweezers. Next, the bone fragment was returned to its place and the surgical layers were sutured. **Results**: This new technique is easy to be done, avoids bleedings and removes only the pineal gland without damage to the remaining encephalon. In addition it makes possible the achievement of a sham surgery, allowing the pineal gland to remain intact. **Conclusion**: The proposed technique intends to facilitate studies aiming to better understanding the complexity and importance of the pineal gland on reproductive and other body systems.

Key words: Pineal Gland. Melatonin. Genitalia, Female. Surgery. Rats.

RESUMO

Objetivo: Apresentar nova técnica para pinealectomia em ratos. **Métodos**: 80 ratos adultos fêmeas (*Rattus norvegicus albinus*) foram pesados e em seguida anestesiados por via intraperitoneal com xilazina e cetamina. Em seguida os animais foram fixados em uma prancha de cortiça e feita uma incisão na pele e no tecido subcutâneo, na região superior da cabeça, evidenciando a junção dos ossos parietais e occipital. Na região do lambda, realizou-se uma perfuração circular, na calota craniana, com o auxilio de uma broca (4 mm) acoplada a um micromotor. Nesse orifício, após a dissecação da dura-mater visibiliza-se a confluência dos seios venosos longitudinal e transverso. Com o auxilio de uma pinça curva esses seios são deslocados, ligados e identificada a glândula pineal, que pode ser removida em peça única. Em seguida, o fragmento ósseo é devolvido ao seu lugar e as camadas cirúrgicas suturadas. **Resultados**: Esta nova técnica é fácil de ser feita, evita hemorragias e só remove a glândula pineal, sem prejuízos para o restante do encéfalo. Além disso, possibilita a realização de uma cirurgia *sham*, visto que a glândula pineal permanece sem alterações. **Conclusão**: Espera-se que a técnica proposta facilite estudos visando a uma melhor compreensão da complexidade e importância da glândula pineal sobre a reprodução e outros sistemas do organismo.

Descritores: Glândula Pineal. Melatonina. Genitália Feminina. Cirurgia. Ratos.

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Introduction

Many aspects of pineal metabolism make this gland an excellent subject for the study of biological rhythmicity. Also, the participation of the pineal gland in the regulation of seasonal, photoperiodic-dependent reproduction has been firmly established; nonetheless, it is not completely understood. Melatonin is produced by the pineal gland[1,2]. Its release is mainly due to pineal secretion, but 25% of melatonin production is of extrapineal origin[1].

Melatonin secretion is elicited by norepinephrine (NE)-driven pulses, which are released by intraparenchymal nerve fibers. This NE-releasing activity and hence the functioning of the pineal gland are activated in the dark environment and inhibited by light. The dark–light conditions are transmitted through the eyes to the suprachiasmatic nucleus, which signals are inhibited by light and are activated in the dark as a consequence of the absence of such inhibition[3]. The majority of suprachiasmatic nucleus neurons respond to retinal illumination with an increased firing rate[3]. The

mechanisms by which melatonin acts on the neuroendocrine systems to affect reproduction are not known. It is thought that melatonin acts directly by affecting the hypothalamic functions involved in the inhibitory regulation of GnRH[4]. The release of the pituitary gonadotropic hormones, FSH and LH, often occurs on a rhythmic basis with the period of release ranging from an ultradian (about 1– 4 hours) to a circadian (about 24 hours) and a seasonal (about 1 year) pattern[5].

In photoperiodic species, the pattern of melatonin secretion by the pineal gland mediates the effects of day length on the seasonal reproductive cycle[3]. In those species, melatonin has a progonadotropic effect, namely increasing FSH concentrations and LH pulses, probably by inhibiting the inhibitory effects of sex steroids on ovulation[6,7,8].

In a number of rodent species (rat and mouse) and other mammals, the preovulatory release of gonadotropins is tightly controlled by a neural circadian clock. These are called nonphotoperiodic species, most of them with short-term periods of pregnancy, which do not obey a seasonal reproductive rhythm, but show a daily circadian rhythm of melatonin release. In rodents, melatonin has marked antigonadotropic properties, such as inhibition of gonadal development, spermatogenesis and androgen production in males, in addition to the absence of follicles and corpora lutea (CL), with interstitial tissue proliferation in female rats[6,9].

The necessity of studying the physiology and the metabolism of the pineal gland and also the biologic rhythm of many species, explains the importance of its removal (pinealectomy). Some authors developed this technique in small animals[10,11]. More recent studies in our laboratories, involving melatonin action on the female reproductive system, demand for a large number of pinealectomized rats.

The pinealectomies performed following the Hoffman and Reiter[10] and Kuszak and Rodin[11], presented some troubles, such as neurologic damage, intracranial hemorrhage and death. In order to solve this problem in removing the gland and maintaining a good clinical condition of the animal after surgery, we modified the technique of pinealectomy. However, it is important to emphasis that there are main challengers in this surgery: 1 - to open the cranial bone without injuring the dura mater and the venous sinuses; 2 - to avoid the bleeding caused by the opening of the cranium bone; 3 - to find the pineal gland and 4 - to remove correctly the pineal gland without damaging the encephalon. Therefore our aim in this paper is to describe the pinealectomy technique that we standardized in our laboratories and discuss some aspects of pinealectomy effects on the female reproductive system.

Methods

Eighty adult female rats (3 months old) Wistar (*Rattus norvegicus albinus*) of the EPM-1 strain were obtained from the Center for the Development of Experimental Models for Medicine and Biology of the Federal University of Sao Paulo (UNIFESP). The animals were housed in individual clear plastic cages under controlled conditions of temperature (22 ± 1 ºC). Standard rat chow and tap water were available *ad libitum*. The rats averaged 345 ± 47 g body weight at the beginning of the study. After an adaptation period of 2 weeks, vaginal smears were taken to determine the cyclicity during a 30-day period. The rats showed repetitive estrous cycles and vaginal smears continued to be monitored throughout the experiment.

The Laboratory Animal Care Committee of Federal University of Sao Paulo approved this study; rats were maintained in accordance with the Guiding Principles for The Care and Use of Animals. The project design was approved by the ethics and research committee of the Federal University of Sao Paulo (Protocol 0233/06).

Vaginal smears were obtained throughout the study using cotton swabs dampened in saline solution. The smears were transferred to histologic slides and stained by the Shorr-Harris method. The four phases of the estrous cycle of the rat (proestrus, estrus, metestrus, and diestrus) were identified. Upon completion 3 months of treatment, all animals were sacrificed during the estrous phase and in the pinealectomized groups, the skulls were opened to ascertain the correct removal of the gland.

Rats were weighted and anesthetized intraperitoneally with 15 mg/kg xylazine and 30 mg/kg ketamine. Trichotomy and antisepsis of the dorsal part of the head are done with 2% iodate alcohol. An incision of approximately 20mm is done on the median line of the parietal bone extending to the occipital crest. After removal of the skin the periosteum scraping is done and the fibrous articulations (serrata type) between the parietal bones and between these and the interparietal bone are exposed (Figure 1, A).

A circular incision is done with the assistance of a low rotation micromotor, maximum 20.000 rpm (NSR, NEVONI) together with a dentist drill engaged in a mandrel. It takes as center the confluence of parietal bone articulations and also the articulations of the parietal with the interparietal bone. It measures 4 mm of diameter. This incision should be done only in the bony part, so that the dura mater is not damaged and there is no rupture of blood vessels. After the incision, the bony fragment is removed and maintained safe wrapped apart in a piece of saline-moistened cotton. In this moment the dorsal longitudinal venous sinus and transverse one are clearly identified below the dura mater (Figure 1, A and D). After that the animal is taken under magnifying glass (PZI-WARSZAWA) that will be used in the next steps.

Using the simple gastrointestinal 5-0 suture line the double closure of dorsal longitudinal sinus is done about 2 mm before the dorsal longitudinal sinus confluence and also before the transverse sinus (Figure 1, B), then the dorsal longitudinal sinus section was performed using the iris scissors and we displace the posterior part of the back (Figure 1, C and E). A little bleeding can occur on occasion, but this can be easily controlled using a cotton compress. In this moment the pineal gland is already visible, standing below the dorsal longitudinal sinus confluence, below the transverse one and between the cerebral hemispheres and the cerebellum. Using adequate calipers, the pineal gland is now picked up, removed and immersed in formaldehyde a 10 %.

After these procedures, the venous sinuses are taken back to their original position, care being taken to avoid the formation of casual anastomosis. The cranium bone fragment is taken back to its initial position and the suture of the skin is done using a polypropylene-0 (Prolene) thread. After termination of the anaesthetic effects the animals quickly recover normal walk.

After surgery, the animals received a single 570 mg/kg dose of prophylactic antibiotic (Pentantibiotic) by the SC route.

Twenty-four hours later the samples (pineal gland) were submitted to conventional processing for histological examination,

included in paraffin and cut with a manual microtome in a thickness of 5µm. The sections were stained with hematoxilin and eosin (H.E). The microscope used for this analysis was the binocular Zeiss (Mod. Axiolab).

Results

Death rate due to the procedure was as low as 6.3% (5 deaths in 80 animals), most probably consequent to anesthetic-induced central depression. In fact, deaths occurred always during the establishment of the surgical stage of anesthesia, and no animals were lost during the operative procedure or at the post-operative period.

Anaesthesia was one of our difficulties to perform in this operative technique. Among the several dose combinations essayed, the best was xylazine: ketamine at a 15:30 mg/kg dose ratio, such as to produce an appropriate and reliable level of anesthesia. The negative point in this kind of anesthesia was the very slow metabolism leading to sleep of more than 40 min. Mainly in cold days, this procedure caused the death of some animals. In order to solve this problem, we started to keep up the animal in warm places during the after surgery. It should be mentioned that the most important is to keep the animal fixed during the surgery period that lasts about 20 minutes.

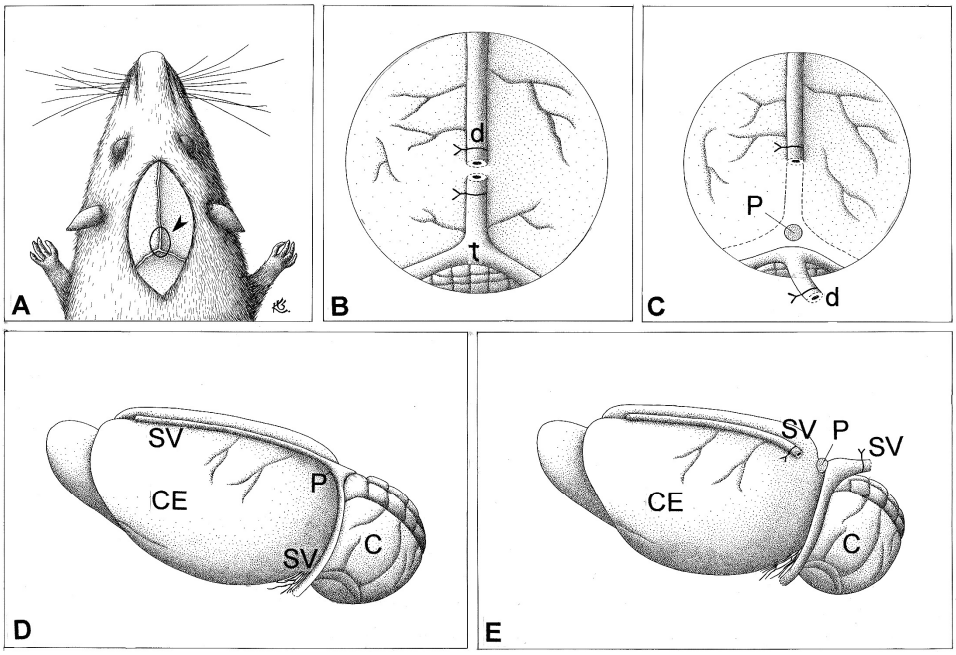

FIGURE 1 - Illustration showing the surgical steps for pinealectomy by direct visual approach in rats. **A -** the arrow shows the localization of the bone circular incision for removing the pineal gland in the rat head; **B -** suture of venous sinus under the dura mater, showing (d) dorsal longitudinal vein and (t) transversal vein; **C -** Pineal gland (p) exposition after the venous sinus sutured and sectioned (d); **D** and **E** - Lateral vision of the encephalic regions: brain (CE), cerebellum (C), venous sinus (Sv) and pineal gland (P). Note that E shows the suture of the venous sinus

The linking of the venous sinus is extremely important to avoid bleeding. It makes easy the access to the glad and also its visibility after the uprising of the dorsal longitudinal venous sinus piece. Some pinealectomy techniques perforate the venous sinus causing extensive hemorrhages[10]. Many kinds of suture threads can be used. We used the gastrointestinal 5-0 thread for internal suture and the polypropylene 3-0 thread (Prolene), because these are the most used in our laboratory.

Discussion

We believe that a hallmark of the new approach to pinealectomy proposed herein is the use of magnifying glasses that facilitates the localization, visibility and removal of the pineal gland. This gland is very small (about 0.5 mm of diameter) and is located between the cerebral hemispheres and the cerebellum. The association of adequate callipers with the magnifying glass makes possible the correct and safe access to the pineal gland.

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Light microscopy evaluations confirmed that the material obtained by this technique was exclusively pineal tissue. In other words, only pineal gland is removed, without taking (or injuring) any adjacent structures.

Several distinctive issues deserve special mention regarding the direct visual approach to pineal gland as proposed herein: 1 – the use of a micromotor to perform the incision on the cranium bone without injuring the dura mater; 2 – the ligation of the venous sinuses, thus avoiding bleeding; 3 – the easy access and visibility of the pineal gland; 4 – the safe removal of the gland; 5 – absence of damage to the encephalon and minimal lesion of dura mater; 6 – the possibility of a sham surgery in order to obtain a highly reliable control group of rats with a fully preserved pineal function; 7 – the very fast recovery of the operated animals due to small injured area.

Previously published data from our laboratory showed that pinealectomy determined changes on the gonadotropin levels, ovarian function, endometrial morphology and embryo implantation in rats[12–15]. In brief, Dardes *et al.*[13] demonstrated a decrease in gonadotropins (LH and FSH) at the early post-pinealectomy period and a disruption in estrous cycle. Soares Jr. *et al*[14,15] revealed that the ovaries in pinealectomized rats had an increase in the number of atretic follicles and interstitial cells. These cells showed hyperactivity features on transmission electron microscopy and morphometric analysis. Also, the pinealectomy determined an increase in estradiol and a decrease in progesterone circulating levels. Moreover, progesterone receptor expression was lower than normal animals. In addition, Dair *et al.*[16] reported that pinealectomy may induce endometrial proliferation and reduce the ovulation, as well as embryo-implantation in rats. Also, some studies have shown that pinealectomized female rats present

vaginal cornification, constant estrus and ovulation failures[12–15,17].

Teixeira *et al.*[12] described that pinealectomy may interfere with trophoblast invasion and the pregnancy. In fact, the process of implantation involves complex interactions and requires a very precise coordination between the establishment of uterine receptivity and the blastocyst activation in rats[18]. This process is primarily dependent on the concerted effects of sex steroids[19]. At the beginning of pregnancy, preovulatory ovarian E2 directs epithelial cell proliferation[20]. Consequently, the uterine receptivity may be influenced by changes of the circulating levels of E. On the other hand, melatonin, main pineal hormone, is needed for the development of important cell structures, such as pinopodes and microvilli in rodent and human endometria[21]. This fact may explain the reduction in the number of blastocyst implantations in the group of pinealectomized animals.

Pinealectomy determines the reduction of melatonin levels as well as the hormone peak during the night. Therefore, many actions related to the process of reproduction represent the largest group of the spectrum of functions identified: regulation of GnRH, gonadal development, spermatogenesis, and androgen production in males; and regulation of follicles, luteal bodies and interstitial tissue proliferation in female rats[17]. Conceivably, studies on hitherto unrecognized or poorly understood aspects of the role played by the pineal gland in the body could benefit from the pinealectomy technique described here.

Conclusion

It is hoped that the proposed technique helps studies aiming to better understanding the complexity and importance of the pineal gland on reproductive and other body systems.

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