Ether as an Anesthetic for Decapitation in the Rat: Gonadotropin Secretion by Subsequently Established Anterior Pituitary Cell Cultures (42866)

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Abstract. **Historically, for the establishment of dispersed anterior pituitary cell cultures, rodents have been killed by decapitation without anesthesia. Because decapitation fails to induce immediate unconsciousness, the American Veterinary Medical Association Panel on Euthanasia has recently recommended that rodents should not be decapitated without previous anesthesia or sedation. Investigators are therefore confronted with the dilemma of wishing to euthanize rodents humanely yet not wishing to potentially compromise experimental data through the use of anesthetics. We present our observations on the effects of diethyl ether anesthesia administered prior to decapitation on the gonadotropin secretory characteristics exhibited *in vitro* by cultured rat anterior pituitary cells. Neither light nor complete (surgical level) ether anesthesia had any statistically significant effect on either luteinizing hormone or follicle-stimulating hormone responsiveness or cell content of luteinizing hormone and follicle-stimulating hormone or DNA content. The data indicate that ether anesthesia would appear to be acceptable for those studies involving subsequent *in vitro* luteinizing hormone and follicle-stimulating hormone secretion.** [P.S.E.B.M. 1989, Vol 190]

Dispersed cell culture systems are frequently utilized as tools in studying pituitary hormone secretion and synthesis. Rodents, in particular the rat, are often used as a source of anterior pituitary cells for these cultures. Investigators have historically avoided the use of anesthetics prior to killing, particularly in neuroendocrine and metabolic regulation studies, for two major reasons. First, the hypothalamic pituitary axis is recognized to be highly sensitive to the stress (1, 2) which might be encountered during the manipulations of the animal necessary to administer the anesthetic. Second, the anesthetic agent itself within the tissues of the animal might compromise experimental data. Decapitation has therefore become the method of choice in many laboratories for killing rats because it has been considered a method of inducing instantaneous unconsciousness and death. However it has been shown, as judged by EEG data, that rats may not lose consciousness for approximately 30 sec subsequent to decapitation (3). Largely based on this one report, the American Veterinary Medical Association Panel on Euthanasia (4) has recommended that rodents be euthanized by decapitation only after being placed under sedation or light anesthesia.

Nazian (5) has reported findings concerning the effects of various anesthetic agents, including ether, on the *in vivo* secretion of several reproductive hormones. This report details our findings concerning the effects of diethyl ether anesthesia administered immediately prior to decapitation on *in vitro* pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) exhibited by dispersed anterior pituitary cell cultures and is addressed to those investigators (ourselves included) who are utilizing decapitation without anesthesia for the purpose of establishing rat anterior pituitary cell cultures.

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Materials and Methods

Animal Procedures. Sprague-Dawley rats (65 days old) in random stages of the estrous cycle were used. Within a given experiment, all animals were of the same birthdate and were obtained from Holtzman (Madison, WI) in a single order. The rats were randomly distributed two per cage and housed under 12-hr light:12-hr darkness conditions with lights on at 6 AM eastern standard time. The animals were handled daily

in order to acclimate them to laboratory auditory and visual stimuli. The experimental procedures utilizing animals were approved by the Institutional Committee on Animal Use to ensure compliance with the American Association for Accreditation of Laboratory Animal Care guidelines.

Animals were killed between 8 and 9 AM by instantaneous decapitation at the hands of an experienced individual; for a given experiment all animals were killed on the same morning. Trunk blood was collected at the time of sacrifice for determination of prolactin. The animals were divided into three groups of 12 rats/group. Diethyl ether (Baker #9249-04) was administered by placing individual animals into gallon glass vessels containing ether-saturated atmospheres. Group 1 received no anesthesia and was euthanized first. The animals of Group 2 received light diethyl ether anesthesia by being placed in the ether atmosphere for approximately 45 sec and Group 3 received exposure for approximately 90 sec. Stages of anesthesia were defined as described by Ben *et al.* (6).

Cell Dispersal. Anterior pituitary tissue from each group was pooled in Dulbecco's modified Eagle's medium (see below) and dispersed utilizing trypsin (GIBCO #610-5090) as described previously (7–9). Culture medium contained charcoal-treated (10, 11) 5% fetal bovine and 5% horse serum and was based in Dulbecco's modified Eagle's medium without phenol red (GIBCO #430-3000 EB); phenol red was avoided because it has been found to exhibit potential estrogenic activity (12–14). The dispersed cells were plated in 6-well culture plates (GIBCO #925-3120 XT) in the ratio of one pituitary equivalent per ml per well. Dispersed cell viability exceeded 90% as indicated by trypan blue exclusion (15).

Luteinizing Hormone-Releasing Hormone (LHRH) Stimulation. At 72 hr in culture the serum-containing medium was removed and replaced with 900 μl of serum-free Dulbecco's modified Eagle medium. LHRH (Sigma #L-7134) was added to duplicate culture wells in 100 μl of 0.01 *M* phosphate-buffered saline plus gelatin (PBS plus gel). At 3 hr the culture media were removed and the cultures covered with 1 ml of PBS; both media and cells were frozen at −20°C to await assay of LH and FSH. The cells were thawed and scraped with a Teflon policeman, homogenized in 1 ml of PBS, centrifuged at 1000*g*, and the supernatant was assayed in order to determine unreleased LH and FSH. The cell pellet from this centrifugation was subjected to DNA assay (see below).

Radioimmunoassay Systems. LH and FSH were estimated in culture media and cell homogenates utilizing reagents provided by the NIADDK Pituitary Hormone Distribution Program. These assay systems have been described in previous publications (7, 16, 17). LH and FSH data are expressed in terms of RP-1.

Blood levels of prolactin circulating at the time of decapitation were also determined utilizing NIADDK reagents. The antibody preparation was S-9, the antigen was I-5, and the reference preparation was RP-3. The data were expressed in terms of RP-1. All radioimmunoassay data for a given hormone were generated from a single assay.

DNA Assay. Cell pellets were subjected to DNA assay so that hormone secretion could be calculated on the basis of the amount of viable tissue present at the time of stimulation. The cell pellets were digested overnight at 37°C in 50 μl of 0.005 *N* NaOH. This volume was then subjected to a fluorescent Hoechst 33258 (Sigma #B-2883) assay as described previously (18). Fluorescence was measured in a Sequoia-Turner model 450 fluorometer utilizing a narrow band 360 filter for excitation and a sharp cut 450 filter for emission.

Statistics. Experiments were repeated three times in duplicate ($n = 6$). After LHRH stimulation, data were first calculated as nanograms of LH or FSH per nanogram of DNA; these data were then plotted as fold increase over basal (for release) or as content after stimulation divided by content before stimulation (for cell content). Data were evaluated vertically (across anesthesia treatments) at a given LHRH dose by one-way analysis of variance (ANOVA); if significant differences were indicated, Duncan's multirange test was employed as a post-test. All values are plotted as mean (±SE); differences of $P < 0.05$ were considered significant.

Results

LHRH-Induced LH Responsiveness. Neither basal nor LHRH-stimulated LH responsiveness was significantly different at either level of anesthesia in comparison to control (Fig. 1A). When nonreleased cellular levels of LH were evaluated subsequent to LHRH stimulation (Fig. 1B), no significant differences were found to exist between any of the treatment groups. Etherization was found to have no significant effect on the amount of DNA per culture.

LHRH-Induced FSH Responsiveness. When LHRH-induced FSH responsiveness was evaluated by ANOVA (Fig. 2A), no significant differences were indicated between the nonanesthetized and either anesthetized group. When nonreleased cellular levels of FSH were statistically evaluated subsequent to LHRH (Fig. 2B), ANOVA indicated no significant differences to exist.

Discussion

Because there is open debate concerning whether the brain of nonanesthetized, decapitated rodents experiences pain during the estimated 30 sec required for EEG inactivity (death) to occur (3), the American Veterinary Medical Association Panel on Euthanasia has recommended that rodents not be decapitated without sedation or light anesthesia (4). Although exception has

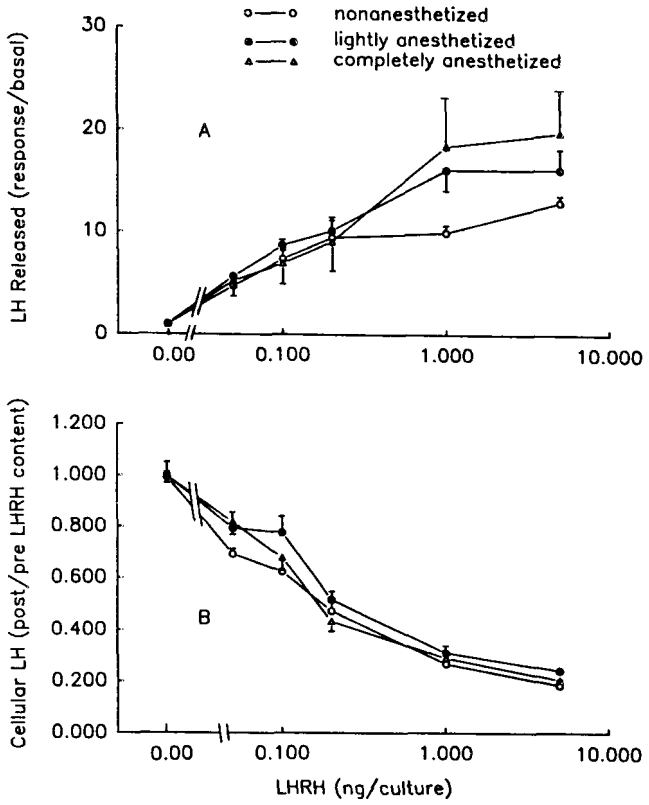

Figure 1. LH responsiveness of anterior pituitary cell cultures established from rats exposed to various levels of ether anesthesia. The cells were exposed to the indicated levels of LHRH for 3 hr; media (A) and unreleased cellular LH levels (B) were then determined by radioimmunoassay. Data are plotted as the mean (±SE) of three experiments repeated in duplicate ($n = 6$).

been taken to this recommendation (19), it exists nonetheless and practicing scientists must give it consideration. Many reports have described *in vivo* effects of anesthesia on various physiologic parameters (4) and Nazian (5) has recently studied the effects of various anesthetics on *in vivo* gonadotropin secretion; however, no reports currently exist describing the effects of anesthesia administered immediately prior to decapitation on the subsequent gonadotropin secretion exhibited by anterior pituitary cell cultures. In the present study, diethyl ether was selected as an anesthetic agent for a number of reasons. It has been pointed out (6) that ether is probably the most effective, safest, easiest to administer, and most economical inhalation anesthetic. Ether also has analgesic action (20) whereas the barbiturates and halothane have only minimal analgesic action. In addition, the presentation and identification of the stages of anesthesia occur more predictably when ether is used than with other anesthetics (20). Because our previous studies have shown hemipituitary incubation systems to potentially yield variable responsiveness to LHRH (21), the current study was conducted utilizing a dispersed cell culture system; such systems have been found to yield highly reproducible responses (17, 22, 23) and to maintain the relative *in vivo* LHRH responsiveness of the donor (7, 17, 24–29).

When circulating prolactin levels in existence at the time of decapitation were determined (data not shown), no significant differences were found between the anesthetized and nonanesthetized animals, confirming the previous results of others that rapid etherization causes no stress-induced elevation of prolactin. Basal LH secretion was not significantly influenced by etherization. Both light and complete ether anesthesia tended to increase LH responsiveness at the two higher LHRH dosages employed; however, variability at higher LHRH levels led to an indication of no statistically significant difference between anesthetized and nonanesthetized as indicated by ANOVA. Nazian (5), studying anesthesia effects in male rats *in vivo*, found that etherization significantly increased LH secretion; however, these effects were observed only in castrated immature rats and no significant effects were observed in intact or castrated adult or intact immature animals. Cellular nonsecreted levels of LH remaining subsequent to LHRH stimulation (Fig. 1B) were found to be insignificantly different at all levels of etherization. Etherization had no effect on the number of cells in culture as indicated by the DNA data (not shown). Basal secretion of FSH was not significantly affected by ether. As

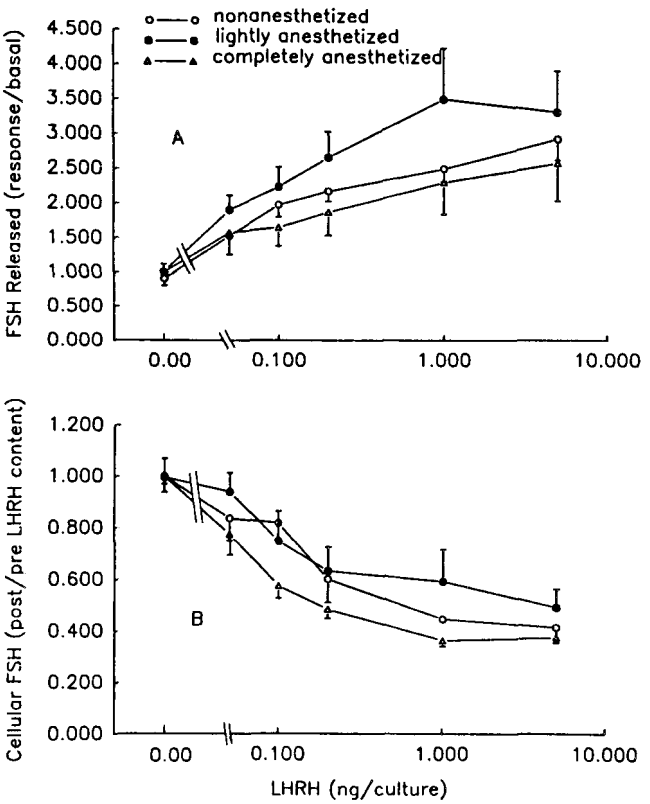

Figure 2. FSH responsiveness of anterior pituitary cell cultures established from rats exposed to various levels of ether anesthesia. Remainder of legend reads as in Figure 1 with the substitution of FSH in place of LH.

with LH, both etherized groups tended to exhibit more variability at higher LHRH levels (Fig. 2A) than did the nonanesthetized group; however, neither light nor complete anesthesia had a statistically significant effect on the FSH response. Nazian (5) has found no significant effect of etherization on FSH secretion *in vivo* in the male rat. Nonsecreted cellular levels of FSH (Fig. 2B) were not significantly affected at any LHRH level by etherization.

In vivo ether anesthesia had no statistically significant effect on the LH or FSH secretory properties of cultured anterior pituitary cells. Etherization would then seem to be an acceptable method for relieving the pain and stress probably associated with decapitation in rats. It should be emphasized that this study has addressed only the potential for modification of LHRH responsiveness *in vitro* in the female rat; the data should not be interpreted or construed to indicate that other parameters of pituitary function would be affected in a similar manner. The data also should not be applied to those investigators who are studying entirely different metabolic processes.

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