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Huperzine-A administration recovers rat ovary function after sympathetic stress

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Abstract

Chronic cold stress affects ovarian morphology and impairs fertility in rats. It causes an ovarian polycystic ovary (PCOS)-like phenotype, which resembles PCOS in women. The mechanism of cold stress action involves increased ovarian noradrenaline (NA) levels, which remain elevated after cessation of cold stress. By contrast, ovarian acetylcholine (ACh) levels are only transiently elevated and returned to control levels after a 28-day post stress period. Because ACh can exert trophic actions in the ovary, we hypothesised that a sustained elevation of ovarian ACh levels by intraovarian exposure to the ACh-esterase blocker huperzine-A (Hup-A) may interfere with cold stress-induced ovarian changes. This possibility was examined in female Sprague-Dawley rats exposed to cold stress (4°C for 3 h day^{-1} for 28 days), followed by a 28-day period without stress. To elevate ACh, in a second group Hup-A was delivered into the ovary of cold stress-exposed rats. A third group was not exposed to cold stress. As expected, cold stress elevated ovarian NA, reduced the number of corpora lutea and increased the number of follicular cysts. It increased plasma testosterone and oestradiol but decreased plasma levels of progesterone. In the Hup-A group, ovarian levels of both, NA and ACh, were elevated, there were fewer cysts and normal testosterone and oestradiol plasma levels were found. However, progesterone levels remained low. Most likely, low progesterone was associated with impaired mating behaviour and low pregnancy rate. We propose that elevated intraovarian levels of ACh are involved in the rescue of ovarian function, opening a target to control ovarian diseases affecting follicular development.

KEYWORDS

acetylcholine, fertility, huperzine-A, polycystic ovary, stress

1 | INTRODUCTION

The ovary contains a delicate network of sympathetic nerves coming from the celiac ganglion, which innervates the ovarian follicles and blood vessels.[1-4]

Exposure of adult rats to cold stress elevates sympathetic activity and increases noradrenaline (NA) plasma levels without affecting corticosteroid levels.[5] This increase in NA also occurs in the ovary, where it produces alterations in follicular development, such as a decrease in secondary follicles and the appearance of follicles with hyperthecosis (ie, a follicular abnormality characterised by a hypertrophied theca cell layer).[6] Follicles with hyperthecosis are responsible for androgen hypersecretion, the first step in the development of ovarian cysts. Likewise, when the exposure time of rats to the cold stress paradigm is increased to 8 weeks, follicles with hyperthecosis and cysts are generated. This implies the formation of the polycystic (PCOS-like) phenotype in the rat ovary.[7-10] Most of these changes in follicular development and steroid biosynthesis are characteristically seen in women with polycystic ovary syndrome (PCOS, the most common ovarian pathology in women during reproductive age.[11,12]

The ovary also receives parasympathetic vagal fibres. Parasympathetic nerve fibres enter the hilar region and are associated with blood vessels but do not directly reach the ovarian follicle.[13] In addition, there is a local cholinergic system, driven by granulosa cells (GCs) of the follicle, which produce acetylcholine (ACh).[14] On functional grounds, it has also been described that GCs increase the production of ACh in response to follicle-stimulating hormone.[15] Both in vivo and in vitro experiments show that human and rat GCs express ACh receptors of the muscarinic subtypes.[16,17] Finally, the enzyme that degrades ACh, ACh-esterase (AChE), which hydrolyses ACh to choline and acetate, has also been characterised in human GCs and in the rat ovary.[18,19]

Previous studies using huperzine-A (Hup-A), a potent, selective and reversible inhibitor of the catalytic activity of the enzyme AChE,[20] indicated that the cholinergic system is functionally active in vivo in rats and in ovarian follicle cultures of a nonhuman primate. Administration of Hup-A increased not only intraovarian ACh, but also the number of small secondary follicles in rats. This implies an increase in initial follicular development.[19] Indeed, Hup-A further elevated the growth of antral follicles and the oocyte in isolated nonhuman primate follicle cultures.[21] In rats, the number of atretic antral follicles and corpora lutea increased, and the number of pre-cystic follicles decreased. This implies an increase in follicular recruitment, and consequently more ovulations. It is noteworthy that this treatment also enhanced fertility.[19]

Such evidence suggests an important role of the local cholinergic system in follicular development and indicates that the intraovarian cholinergic system can be targeted to pharmacologically regulate ovarian function. We hypothesised that manipulation of this system may be useful when the ovary presents functional alterations of follicular development, such as in PCOS-like phenotype generated by cold stress. Therefore, the present study aimed to examine the question of whether the PCOS-like phenotype induced by cold stress can be antagonised by a long-lasting elevation of intraovarian ACh, by using intraovarian exposure to Hup-A during cold stress.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

In total, 60 female Sprague-Dawley rats weighing 250-300 g were used in all of the experiments. All animals were housed in a maintenance room under a 12:12 hour light/dark photocycle at 20°C. The animals had food and water available ad lib. The first 30 rats were divided into three groups of 10 animals each. Two of these groups of 10 rats were exposed to cold stress for 28 days (3 hours day^{-1} at 4°C, 5 days $week^{-1}$), whereas the other group of 10 rats was kept at room temperature (Control). After this period, the 30 rats were unilaterally ovariectomised and the right ovary removed and saved for subsequent analysis. After ovariectomy, a mini-osmotic pump filled with Hup-A (10 μmol L^{-1} in saline) was implanted into the bursal cavity of the remaining ovary. This procedure has been described and validated previously.[19] Briefly, for this procedure, a miniosmotic pump (Alzet model 2004; Durect Corporation, Cupertino, CA, USA) was filled with Hup-A. A 10-cm cannula was attached at the flow moderator of the minipump. The minipump was implanted s.c. and the cannula was introduced to the peritoneal cavity by the dorsal region of the rat and the cannula was implanted into the bursal cavity and attached to the wall of the bursa with surgical glue. Another group was subjected to the same procedure including the cannula and implantation to the intrabursal cavity, although the cannula was sealed at the end of the miniosmotic pump which was not used (Control) (Table 1). In five rats of each condition, a piece of the ovaries obtained during ovariectomy was used to measure ACh levels at the end of 28 days of stress, and this was procedure also performed for a piece from the contralateral remaining ovary after the 28-day post-stress period or Hup-A period. In the other five rats of each group, the ovary was fixed in Bouin's fixative for morphometric analysis of follicular development (Table 1).

During the study period, the groups of rats were examined by daily vaginal smears for oestrous cycle regularity. The results are presented as the number of oestrous cycles with the following

TABLE 1 Experimental groups of rats

		Age and procedure			
n	Group	60 days old:	88 days old:	116 days old:	140 days old:
		Stress	Hup A minipump	Euthanasia (EU)	Mate with male
10	Control	None	None	EU	–
10	Stress	Cold stress	None	EU	–
10	Stress – Hup A	Cold stress	Hup A minipump	EU	–
10	Control	None	None	–	Rate of fertility
10	Stress	Cold Stress	None	–	Rate of fertility
10	Stress – Hup A	Cold stress	Hup A minipump	–	Rate of fertility

Note: Number of rats for the experimental groups used to study ovary function and fertility. Hup-A, huperzine-A.

stages: pro-oestrus (P), oestrus (E) and dioestrus (D). Control rats presented regular 4-day oestrous cycles.[22,23] A retrospective evaluation of the data from our animals indicated that all control rats from our colony show a range of 60%-80% of oestrous cycles (considering short cycles of 4 days and long cycles of 5 days).[10,24]

At the end of the experiments, the rats were killed by decapitation, and the ovaries and plasma were collected. Decapitation was carried out according to the AVMA Guidelines for the Euthanasia of Animals (2020 Edition) [25] and was performed by specialised personnel. The procedure was approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the University of Chile (Protocol number: CBE2016-13) and complied with National guidelines (CONICYT Guide for the Care and Use of Laboratory Animals). The age at the time of death is presented in Table 1.

2.2 | Morphometric analysis

Ovaries were fixed in Bouin's fluid, embedded in paraffin, cut into 6 μm sections, and stained with haematoxylin and eosin. Morphometric analyses of whole ovaries were performed as described previously[26,27] using four ovaries per group for stress and stress-Hup-A groups and five ovaries for the control group. All follicular structures were followed through all sections and counted in every fifth slice. Briefly, primordial follicles were those with one oocyte surrounded by flattened GCs. Primary follicles exhibited one layer of cubical GCs and secondary follicles had no antral cavity but two or more layers of GCs. Antral follicles were those with more than three healthy GC layers, the antrum and a clearly visible nucleus of the oocyte. Atretic follicles had more than 5% of cells with pyknotic nuclei in the largest cross-section and exhibited shrinkage and an occasional breakdown of the germinal vesicle. Pre-cystic follicles were large follicles with or without oocyte, containing four or five plicate layers of small, densely packed GCs surrounding a very large antrum with an apparently normal thecal compartment. Cystic follicles were devoid of oocytes and displayed a large antral cavity, a well-defined thecal cell layer and a thin (mostly monolayer) GC compartment containing apparently healthy cells.[27] All of these abnormal follicular structures were grouped as cystic structures.

2.3 | Fertility

The second group of 30 rats was divided in three subgroups. Twenty adult rats were exposed to 28 days of cold stress (3 hours day^{-1} at 4°C, 5 days $week^{-1}$) and another 10 were maintained at room temperature (Control). After this period, all rats were unilaterally ovariectomised. After ovariectomy, 10 of the stressed rats were implanted with a mini-osmotic pump containing Hup-A (10 μmol L^{-1}, in saline) and the other half without miniosmotic pump (28 days post stress) (Table 1). Both the 10 control and 10 stressed rats were implanted with the cannula (see above). Twenty-eight days post-pump implantation, the 30 rats were mated with males of proven fertility to evaluate female fertility. This was expressed as the rate (in percentage) between the number of rats that became pregnant and had offspring and the rats that did not become pregnant). Furthermore, uterine implantation sites and the number of pups were evaluated.

2.4 | Mating procedure

Rats were checked for oestrous cyclic activity to determine the pro-oestrus day. On the night of the pro-oestrus phase (when ovulation occurs), the rats were mated with males of proven fertility. The next morning, the rats were checked for the presence of a vaginal sperm plug. Rats with a vaginal plug were assigned as day 0 of pregnancy. If the rats did not show signs of pro-oestrus (as seen in stressed rats) during a period of 2 weeks, they were permitted to stay each night for 2 weeks with the males. They were checked every morning for a sperm plug. If there was no sperm plug after 2 weeks, the rats were assumed to not to have accepted the male and they were designated as infertile.[19,28]

2.5 | Number of offspring and implantations in uterine horns

During the day of birth, the number of live pups was counted and pups were weighed. On day 4 after delivery, the mothers were killed and the uterine horns were exposed to view the number of implantation sites in the uterine horn and compared with the number of pups born.[19,28]

2.6 | Quantification of intraovarian levels of ACh and AChE-activity

As described previously,[10,19] the ovary was homogenised in 10 volumes of phospated-buffered saline in ice. ACh determination was performed in the homogenate using the Amplex® Acetylcholine/Acetylcholinesterase Assay Kit (Invitrogen, Eugene, OR, USA) in accordance with the manufacturer's instructions. Briefly, ACh is hydrolysed by AChE, the choline formed is then oxidised by choline oxidase, and the H_2O_2 resulting from this reaction interacts with Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) in the presence of horseradish peroxidase to form the highly fluorescent resorufin. The results are expressed as the total amount (μmol) of ACh per ovary. The minimal detectable value for AChE was 0.002 U mL^{-1} and, for ACh, it was 0.3 μmol L^{-1} (range 0.3-100 μmol L^{-1}).

2.7 | Plasma levels of steroid hormones

At the end of all the experiments, plasma was collected and kept frozen until analysis. Plasma levels of steroid hormones

testosterone (T), oestradiol (E_2) and progesterone (P_4) were measured. Serum levels of T, E_2 and P_4 were determined by an enzyme immunoassay (EIA) in accordance with the manufacturer's instructions (Alpco Diagnostic, Windham, NH, USA). Intra- and interassay variation was less than 5% for E_2, less than 6% for T and less than 5% for P_4. The minimal detectable values were 10, 0.02 and 0.1 ng mL^{-1}, respectively.

2.8 | Plasma levels of luteinising hormone (LH)

At the end of each experiment, plasma was collected and kept frozen until analysis. Plasma levels of LH were determined by EIA in accordance with the manufacturer's instructions (Alpco Diagnostic). Intra- and interassay variation was less than 1%. The minimal detectable values were 0.2 IU L^{-1}.

2.9 | Statistical analysis

Data are expressed as the mean ± SEM To examine statistical differences between two groups, we used two-tailed Student's *t* test. To analyse differences between several groups, we used one-way ANOVA followed by Tukey's post-hoc test. To analyse difference between proportions we used a chi-squared test (PRISM; GraphPad Software Inc., San Diego, CA, USA). The number of animals for all of the experiments was calculated as the minimum number of animals according to the variability of the experimental procedures and the intrinsic variation between them. The minimum number of animals was calculated according to:[29]

$$n = \frac{2\,(Z\alpha + Z\beta)^2 \times S^2}{d}$$

where n is the number of animals for each condition, S is the standard deviation, d is the difference needed to obtain statistical significance, $Z\alpha$ is the the probability of type I error (significance) and $Z\beta$ is the the probability of type II error (power). In the experiments to determine NA, ACh and AChE activity, as well as the determination of plasma hormones, we propose that $\alpha = 0.05$ (the probability of finding a statistically significant difference is 0.05) and $\beta = 0.3$ (the probability of having a difference between the populations is 0.3), S (the intrapopulation variation) is 2 and d (the smallest difference in the population) is 4.9. Therefore, to obtain a statistically significant difference of $P < 0.05$ we need to use five animals per study group. The same analysis was carried out to determine the ovarian structures, which, because of the variability of the technique, is lower and therefore the necessary number of animals is equal to 4. On the other hand, for fertility experiments, there is a greater variability of the sample such that the required number of animals increases to 10.

3 | RESULTS

3.1 | Effect of Hup-A after stress on ovarian acetylcholine levels

Ovarian ACh levels are shown in Figure 1. As reported previously,[10] we found an increase in ACh ovarian levels at the end of the stress period. Within 28 days after cessation of the stress period, ACh returned to control levels. An increase over the stress-induced levels of ovarian ACh was found after locally administrated Hup-A, indicating that, after stress, Hup-A was effective and blocked ovarian AChE activity.

3.2 | Effect of Hup-A after stress on the follicular dynamics of ovary follicular development

As expected,[10] cold stress produced long-term changes in follicular development and increased ovarian cysts.[7-10] Figure 2 shows a representative image of central slices of rat ovaries of all experimental groups. Twenty-eight days of chronic cold stress exposure resulted in increased numbers of ovarian cysts and decreased numbers of corpora lutea (Figure 2B). The presence of Hup-A during the 28-day post-stress period reversed the morphological changes to a control phenotype, such that Hup-A treatment decreased follicular cysts and increased the number of corpora lutea, Figure 2C).

The quantitative analysis revealed a trend to decrease ($P < 0.06$) in the percentage of secondary follicles in the stress group but a significant increase after Hup-A exposure (Figure 3A). There was statistically significant decrease in the proportion of healthy antral follicles and there were more atretic antral follicles (Figure 3B). Stress increased the number of follicular cysts and Hup-A exposure decreased the number of follicular cysts increased by stress (Figure 3C). Stressed rats presented a decrease of corpora lutea (Figure 3D), although these levels recovered to those of normal (control) rats when Hup-A was administrated during the 28 days after stress exposure. In the Hup-A group furthermore, the numbers of corpora lutea returned to control levels (Figure 3D). The evaluation of the size of corpora lutea, aiming to discriminate between new (bigger size) and old (small size) corpora lutea, showed that cold stress exposure significantly decreased the proportion of bigger corpora lutea (> 700 μm) (Figure 4) and Hup-A reversed the changes and caused an increase in the number of bigger sized (new) corpora lutea (> 700 μm) (Figure 4).

3.3 | Effect of Hup-A after stress on plasma levels of ovarian steroids

Plasma levels of P_4 were significantly decreased after stress (Figure 5A) and both T and E_2 plasma levels were significantly

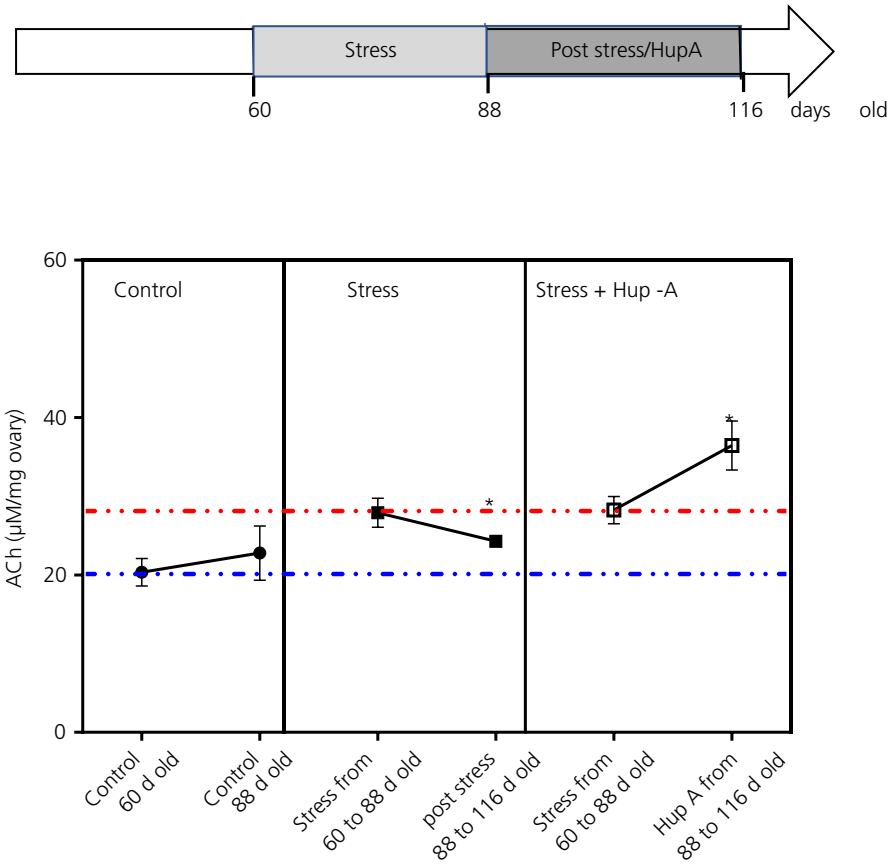

FIGURE 1 Acetylcholine (ACh) levels in the rat ovary. The blue line marks the ACh control levels. The red line marks the ACh levels at the end of the stress period. The groups correspond to control [without stress and without huperzine-A (Hup-A)], 28 days of cold stress exposure followed by a 28-day post-stress period and 28 days of cold stress exposure followed by Hup-A local treatment (n = 5 for each group); data are the mean ± SEM. Asterisks denote statistically significant differences (*$P < 0.05$)

increased over control values (Figure 5B,C), without differences in the T/E_2 ratio (data not shown). Intraovarian Hup-A treatment did not modify the lower levels of P_4 (Figure 5A), although it restored T (Figure 5B) and E_2 plasma levels (Figure 5C).

3.4 | Effect of Hup-A after stress on oestrous cyclicity and fertility

Figure 6A shows a representative view of the cycles of the groups throughout the experimental periods. Stressed rats presented many periods, without regular cyclic activity. The data were also corroborated by a decrease in the number of regular oestrous cycles occurring during the post-stress period (Figure 6B). Intraovarian Hup-A treatment restored the oestrous cyclicity (Figure 6B). In this group, there were no detectable changes in plasma LH levels (Figure 6C).

In Table 2, we summarise the general characteristics of the animals during mating and fertility testing. Control rats presented the expected mating behaviour. When the female was exposed to a fertile male during the night of pro-oestrus, we found that, in 80%, a sperm plug was present the next day, indicating copulation. In the stress group, we observed a sperm plug in only one of the 10 rats. The remaining nine rats accepted the male only when they were exposed to it in a continuous fashion and housed together for 2 weeks. During that time, mating, as indicated by a sperm plug, was checked daily. Of these nine rats, only four became pregnant and had live pups. In all these stress-exposed rats, we observed the same number of pups per animal as in controls (9.8 in the stress group vs 9 in the control group). Hup-A, when applied to the group of stressed rats for 28 days did not improve mating frequency. We found spontaneous mating in one of 10 rats and three of the remaining rats accepted the male after continuous exposure to the male. We did not find any changes in the average number of implantation points or live pups, compared to stressed or control rats.

4 | DISCUSSION

We previously found that local application of the AChE blocker Hup-A to the rat ovary had a stimulatory effect on follicular development, ovulation and enhanced fertility[19] (see also introduction). We concluded that these actions are consequences of elevated intraovarian ACh. We now find that the AChE blocker Hup-A, when applied to the ovary, after exposure to cold stress, resulted in a sustained elevation of intraovarian ACh and thereby was able to prevent the majority of the detrimental morphological changes in the rat ovary provoked by this type of sympathetic stress. Although Hup-A re-established ovulation, it did not elevate low P_4 levels to normal levels and consequently failed to restore normal fertility, at least during the duration of the observation period of the present study. The results of our study indicate that, although Hup-A restored the ovulatory function, female mating behaviour remained impaired and at the same level as the stress group.

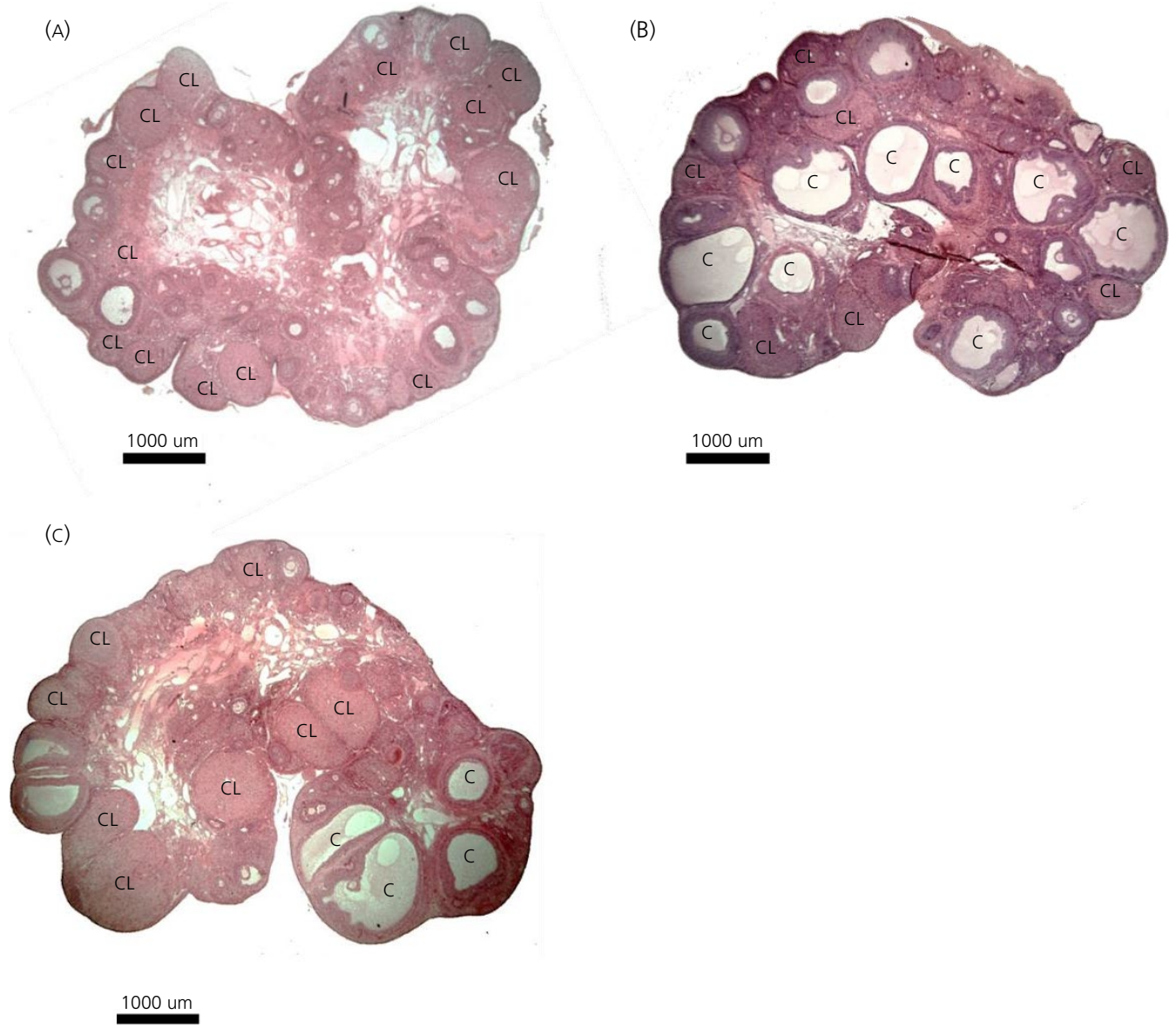

FIGURE 2 (A) Representative picture of a control ovary, (B) an ovary exposed to 28 days of cold stress followed by a 28-day post-stress period and (C) an ovary exposed to 28 days of cold stress followed by 28 days of huperzine-A (Hup-A) local administration. In the images, corpora lutea (CL) and cysts (C) are marked

As previously reported, exposure to 28 days of cold stress increased intraovarian NA levels and induced a PCOS-like phenotype in rat, characterised by ovarian cysts and hyperandrogenism.[7-10] The rapid decrease of the content of ovarian ACh after the end of stress could be the result of higher AChE activity, as suggested previously. Thus, ACh is not available to interact with the cholinergic receptors in the ovary.[10] Increased levels of ovarian ACh can be achieved by blocking the AChE activity with Hup-A, as reported earlier in the normal rat.[19] In the present study, we tested whether a similar approach is effective in rats with decreased fertility linked to stress conditions responsible for the PCOS-like phenotype.

Cold stress exposure caused alterations in follicular development of the rat (increased cysts, decreased corpora lutea), as expected.[7-10] The administration of Hup-A, possibly via increased ACh ovarian levels, reversed all follicular alterations, prevented cyst formation and restored ovulation, as strongly supported by the appearance of new corpora lutea. We recently reported that a similar intraovarian administration of Hup-A to normal adult rats increased ovarian ACh[19] and fertility in rats. Hup-A applied to cultured follicles in a nonhuman primate had trophic effects and enhanced growth of follicles and the oocyte.[21] The presented results show that such trophic actions are also observed under stress conditions in rats.

Plasma levels of steroids closely follow the development of follicles and corpora lutea in the ovary and T levels specifically mirror the development of follicular cysts. Stress elevates androgen levels[8-10] and cyst formation.[30-32] The increase in T would expose GCs to a greater amount of substrate. Consequently, activity of the enzyme P-450 aromatase, which synthesises E_2, likely explains the observed increase in this hormone. Increased T is also related to a higher rate of follicular atresia and to anovulation in rats.[33] Thus, high levels of

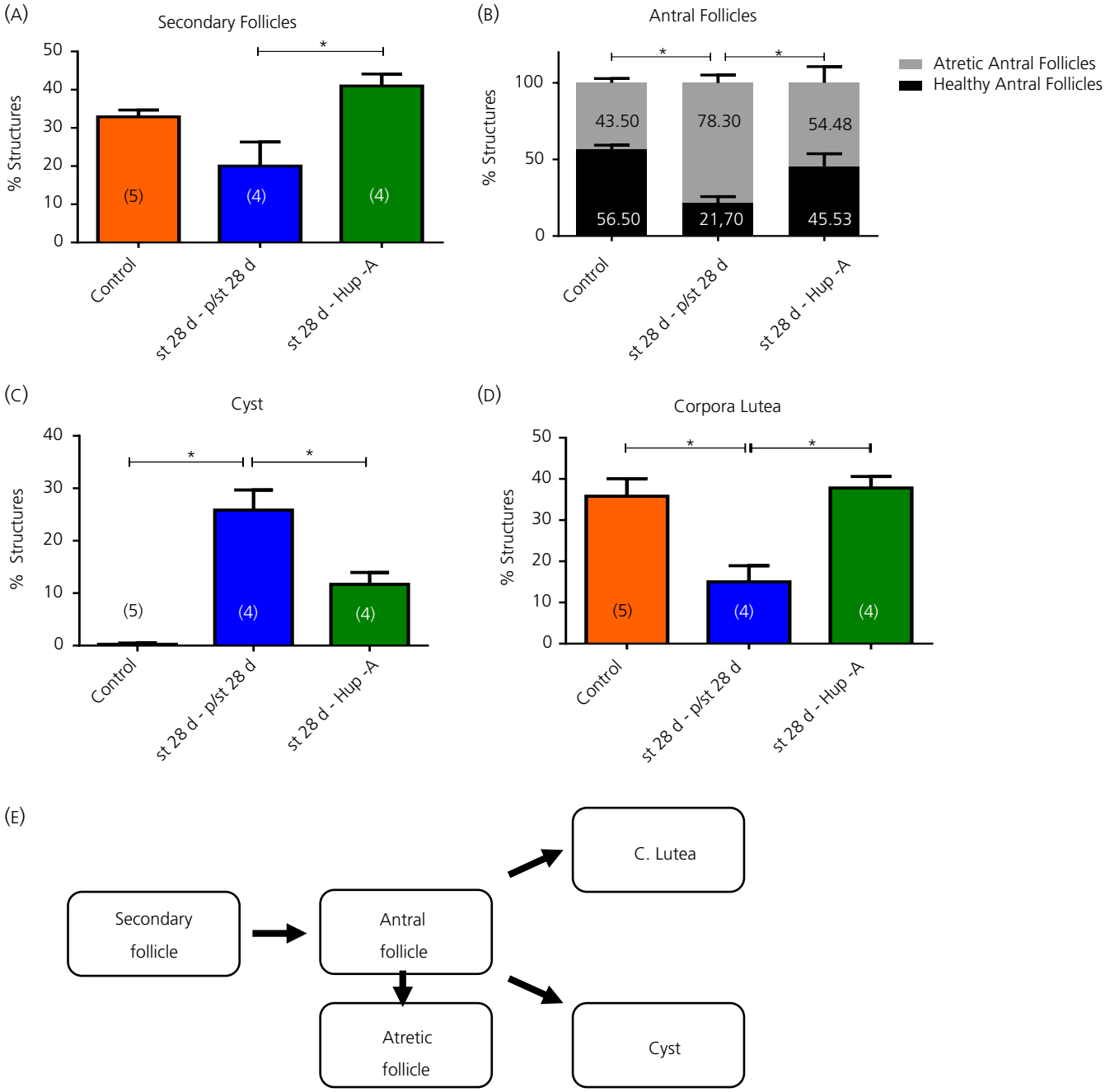

FIGURE 3 (A) Secondary follicles (one-way ANOVA, $F_{2,10} = 6.790$, $P = 0.013$), (B) Percentage of healthy and atretic antral follicles (chi-squared test, $P < 0.05$) and (C) corpora lutea (one-way ANOVA, $F_{2,10} = 10.17$, $P = 0.003$) and (D) cysts (one-way ANOVA, $F_{2,10} = 30.48$, $P < 0.0001$). (E) Scheme of follicular and cyst development. The groups correspond to control, 28 days of cold stress exposure followed by a 28-day post-stress period (Stress 28d + post stress 28d) and 28 days of cold stress exposure followed by huperzine-A (Hup-A) local treatment (Stress 28d + Hup-A 28d) (Control group n = 5, Stress 28d + post stress 28d and Stress 28d + Hup-A 28d n = 4); data are the mean ± SEM. Asterisks indicate statistically significant differences

NA in rats subjected to stress, via T, can contribute to the increase in follicular atresia. P_4 is synthesised by corpora lutea and a decrease in this hormone indicates a decrease in ovulation rate and hence fewer corpora lutea, or impaired function of corpora lutea. Although corpora lutea can be counted, evaluation of impairment of luteal steroidogenesis would require additional studies.

Changes in plasma levels of the steroids, after the intraovarian administration of Hup-A, especially the decrease of the elevated T and E_2 plasma levels present in the stress group, correspond to the fact that only few cysts were found in the morphological analysis. However, Hup-A did not restore the plasma level of P_4, which is mainly produced by the corpora lutea. Adequate levels of P4 in oestrogen-primed rats facilitate proceptivity, sexual receptivity[34] and implantation of the fertilised egg.[35] Progesterone can be derived from non-luteal as well as luteal sources in rats and guinea pigs.[36,37] The clear decrease in copulation events found in the stressed group

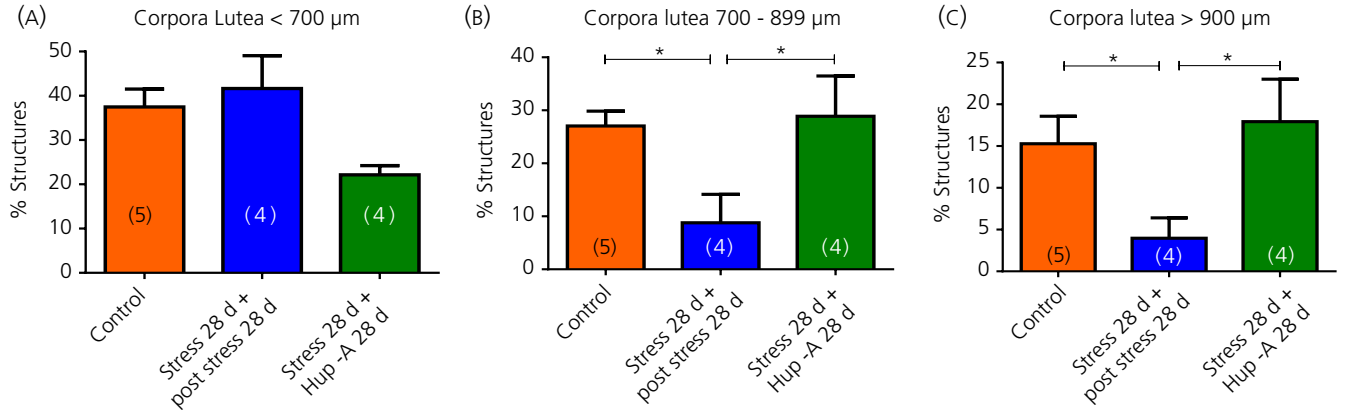

FIGURE 4 (A) Corpora lutea < 700 µm of size, (B) corpora lutea between 700 and 899 µm in size (one-way ANOVA, $F_{2,12} = 3.91$, $P = 0.049$) and (C) corpora lutea > 900 µm in size (one-way ANOVA, $F_{2,12} = 4.88$, $P = 0.033$). The groups correspond to control, 28 days of cold stress exposure followed by a 28-day post-stress period (Stress 28d + post stress 28d) and 28 days of cold stress exposure followed by huperzine-A (Hup-A) local treatment (Stress 28d + Hup-A 28d) (Control group n = 5, Stress 28d + post stress 28d and Stress 28d + Hup-A 28d n = 4); data are the mean ± SEM. Asterisks denote statistically significant differences (*$P < 0.05$)

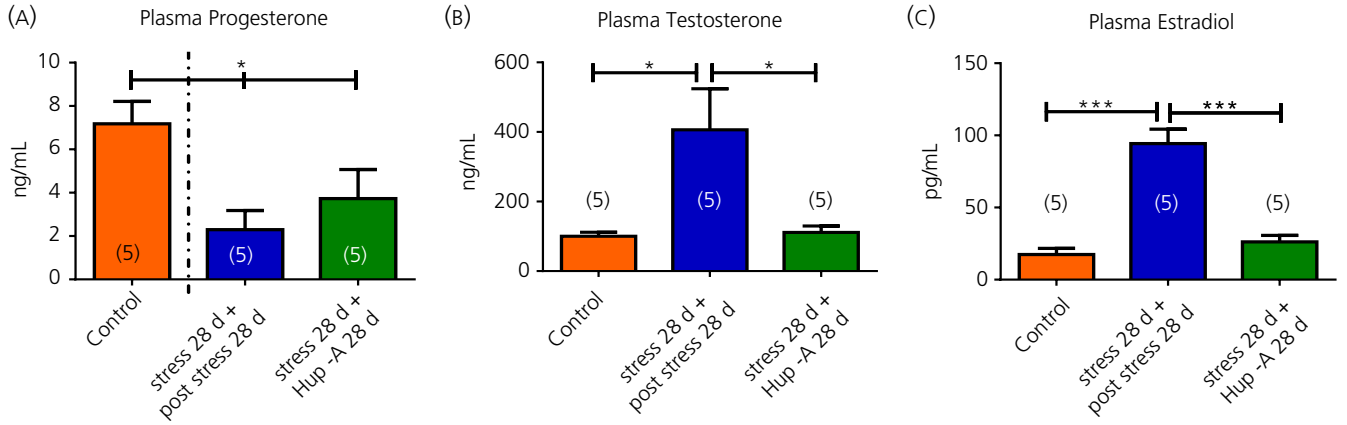

FIGURE 5 (A) Plasma levels of progestrone (P_4) (one-way ANOVA, $F_{2,12} = 4.681$, $P = 0.034$), (B) testosterone (T) (one-way ANOVA, $F_{2,12} = 6.289$, $P = 0.013$) and (C) oestradiol (E_2) (one-way ANOVA, $F_{2,12} = 38.54$, $P = 0.0001$). The groups correspond to control, 28 days of cold stress exposure (Stress 28d + post stress 28d) and 28 days of cold stress exposure followed by a 28-day period post stress (Stress 28d + Hup-A 28d). Hup-A, huperzine-A. T/E_2 ratios were not different between the groups (n = 5); data are the mean ± SEM. Asterisks denote statistically significant differences (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$)

and in the group of stressed rats supplemented with Hup-A implies the possibility that autonomic sympathetic nerve control activated by cold stress and originating from the magnocellular neurones of the paraventricular area of the hypothalamus[38,39] could affect neurones involved in the control of sexual behaviour. Impairment of female sexual behaviour, specifically proceptivity/sexual receptivity, has been associated with the action of P_4 in the hypothalamus of female rats.[40-42] It is noteworthy that stress may impair oocyte quality in women, including growth and maturation and may even cause ageing of oocytes.[43] Such a possibility remains to be examined in rats exposed to cold stress.

With respect to the cause of low P_4, it is possible that the corpora lutea developing from the ruptured follicles were functionally immature, with a reduced capacity to produce steroids. Follicles in the transition to follicular cysts, called type III follicles that can rupture and transform to nonfunctional corpora lutea, were described.[44] Such follicles are precursors of follicular cysts. They are also present in the ovaries of rats exposed to cold stress,[44-47] and therefore we cannot rule out that only partially functional corpora lutea resulted, which would be unable to fully restore P_4 levels. A further possibility, although, to our knowledge, not examined for the female or specifically for luteal cells, is that elevated NA may impair steroidogenesis. In the male rat, such actions of elevated catecholamines are described and resulted in reduced steroid production in Leydig cells.[48] Such a possibility remains to be examined in the rat ovary.

A further important piece of information is provided by the fact that the oestrous cycle apparently became normal upon administration of Hup-A. The evaluation of the cycle is solely based on the cell types found by vaginal smears, which are a consequence of fluctuations of steroid hormones and gonadotrophins.[3,49,50] The cycle is therefore a general indicator of normal functionality of the hypothalamic-pituitary-gonadal axis. Indeed, normal LH levels further support the

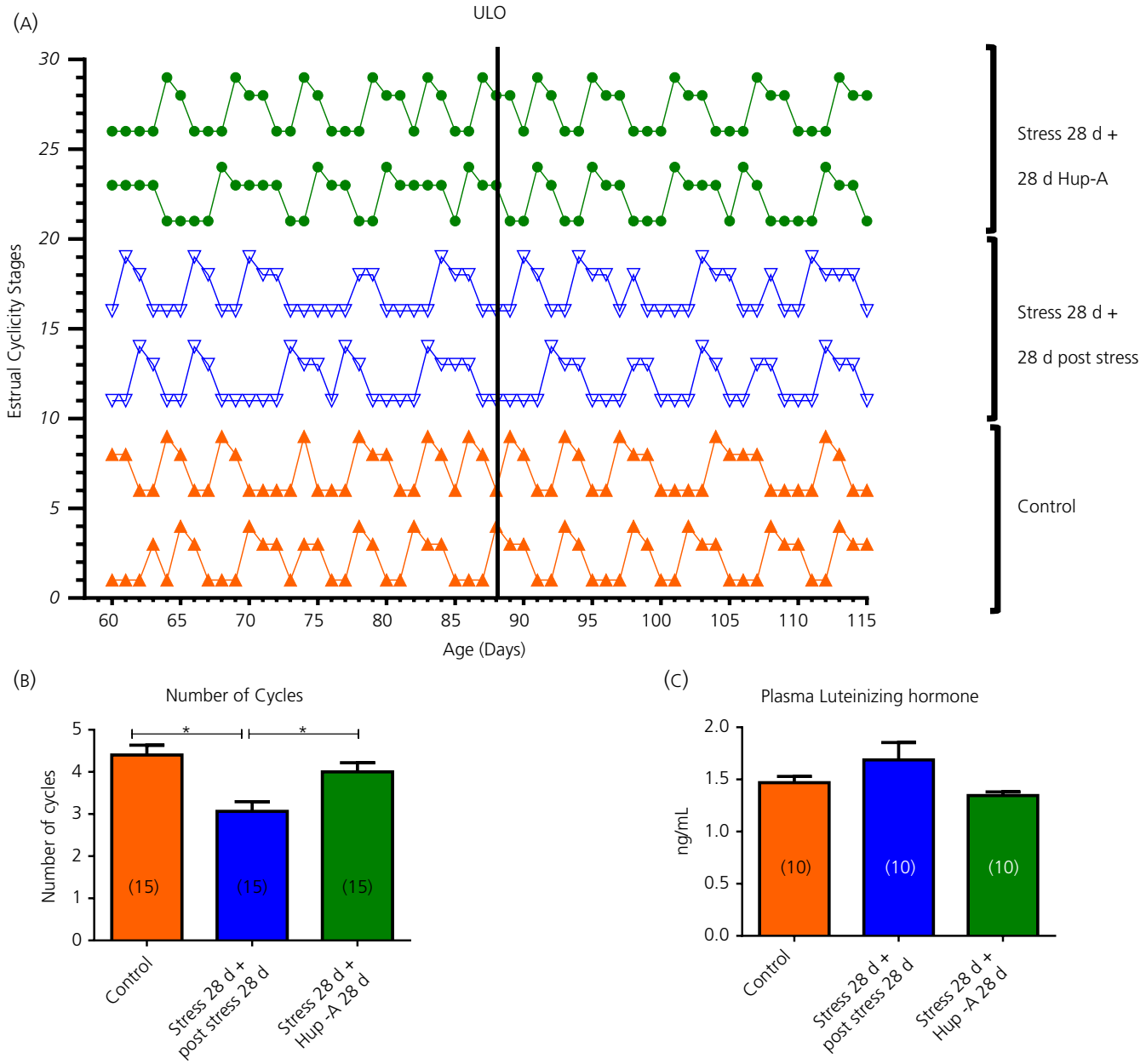

FIGURE 6 (A) Representative graphic of oestrous cyclicity of two rats for each group. The oestrous cyclicity were followed during all periods. (B) Number of cycles during control (red) or Stress/post stress (blue) and Stress/huperzine-A (Hup-A) (green) period (one-way ANOVA, $F_{2,42} = 9.066$, $P = 0.0005$). (C) Plasma levels of luteinising hormone. The groups correspond to control, 28 days of cold stress exposure followed by a 28-day period post stress (Stress 28d + post stress 28d) and 28 days of cold stress exposure followed by Hup-A local treatment (Stress 28d + Hup-A 28d) (n = 15 for each group); data are the mean ± SEM. Asterisks indicate statistically significant differences (*$P < 0.05$)

TABLE 2 General characteristics of fertility parameters in rats

Condition	n	Infertile	Spontaneous mating	Induced mating	Number of rats with implantation points	Average number of implantation points per rat	Average number of pups born per rat
Control	10	2	8	0	8	9	8.0
Stress	10	5	1	4	5	9.8	8.6
Stress + Hup-A	10	6	1	3	4	9.2	9.0

Note: The stress group corresponds to the rats cold-stressed for 28 days and then maintained for a 28-day post stress period with minipumps filled with saline, delivered directly to the ovary. The stress + Hup-A group corresponds to the rats stressed for 28 days and maintained during the 28-day post stress period with a minipump, containing Hup-A solution, delivered directly to the ovary. Hup-A, huperzine-A.

conclusion that the hypothalamic-pituitary-gonadal axis became normalised after Hup-A treatment. Normalisation of the oestrous cycle, judged by vaginal smears, allows the conclusion that, although lower, the levels of P_4, together with otherwise normal E_2, were sufficient to permit changes of the vaginal epithelium indicative of oestrous cyclicity. Although other actions of Hup-A, which were previously reported in neurones, cannot be ruled out completely,[51] the changes induced by Hup-A are most likely a consequence of its action to inhibit AChE; elevated ACh could then bind to cholinergic receptors in the ovary. The presence of muscarinic receptors in the ovary has been described in rats, and M1 participates in the ovulation process in the rat.[17] In addition, in other animal models, such as nonhuman primates, and in human GCs, receptors for ACh have been described[52] and growth promoting actions were described upon activation of these receptors.

Taken together, the data demonstrate that pharmacological inhibition of AChE with Hup-A, and the resulting increase in ovarian ACh, improved most of the detrimental consequences on ovarian function induced by cold stress. This indicates that the intraovarian cholinergic system has important, yet not fully known functions. Further studies are now required to examine the mechanisms involved. The Hup-A induced reduction in ovarian cysts, as well as the reduction of elevated T levels caused by hyper-noradrenergic stimulation typical of the PCOS-like phenotype, indicates that controlled regulation of intraovarian ACh could be a new pharmacological approach for reversing the PCO condition.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Raul Riquelme: Formal analysis; Investigation; Methodology; Software; Writing – original draft. **Freddy Ruz:** Formal analysis; Supervision; Visualisation. **Artur Mayerhofer:** Conceptualisation; Formal analysis; Investigation; Validation; Writing – review & editing. **Hernan E. Lara:** Conceptualisation; Data curation; Funding acquisition; Project administration; Validation; Writing – review & editing.

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