Effects of low dose capsaicin (CAP) on ovarian follicle development in prepubertal rat

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SUMMARY

This study was carried out to investigate effects of low-dose capsaicin (CAP) treatment on follicular development in prepubertal rat ovaries. Eighty female Sprague-Dawley rats (21 days old) were randomly divided into 3 groups according to treatment: whereas the first group received no treatment (control A, n = 5 x 4), rodents of the second group (group CAP, n = 10 x 4) were subcutaneously injected with CAP diluted with solvent (10% ethanol, 10% Tween 80, and 80% distilled water) at 0.5 mg/kg/d and those of the third group were treated with vehicle only (control B, n = 5 x 4). After euthanasia of 10 controls (5 from the group A and 5 from the group B) and 10 CAP-treated rats by ether inhalation at 6, 9, 12 or 15 days, apoptosis was investigated in ovarian follicles by the TUNEL method and by evidence of active 3 caspase expression throughout immunohistochemistry and western blot while cell proliferation was assessed by Ki67 immunohistochemistry. Follicular atresia was observed in the antral follicles (types D and E) of all ovary sections but, in the CAP-treated group, the apoptotic indexes coupled to atresia were significantly lower compared to controls particularly on Days 9 and 15 and the active caspase 3 accumulation in cytoplasm was also significantly depressed. By contrast, follicle proliferation indexes were exacerbated in treated rodents compared to controls on days 12 and 15. The results indicate that the administration of low dose CAP interferes in the ovarian follicular development by partially inhibiting atresia-coupled apoptosis and promoting cell proliferation, probably throughout the release of some neuropeptides present in the capsaicin-sensitive sensory neurons.

Keywords: Capsaicin, ovary follicles, rat, pre-puberty, apoptosis, cell proliferation, TUNEL, caspase 3, Ki 67.

RÉSUMÉ

Effets de la capsaïcine (CAP) à faible dose sur le développement des follicules ovariens chez la ratte pré-pubère

Cette étude a été réalisée dans le but d'étudier les effets d'une faible dose de capsaïcine (CAP) sur le développement des follicules ovariens chez la ratte pré-pubère. Au total, 80 rattes Sprague-Dawley (âgées initialement de 21 jours) ont été aléatoirement réparties en 3 groupes selon le traitement entrepris : alors que le 1er groupe n'a reçu aucun traitement (contrôle A, n = 5 x 4), les rongeurs du 2ème groupe (groupe CAP, n = 10 x 4) ont été injectés par voie sous-cutanée par la CAP diluée dans 80 % d'eau distillée, 10 % d'éthanol et 10 % de Tween 80 à la dose de 0.5 mg/kg/jour et ceux du 3ème groupe ont été traités par le solvant uniquement (contrôle B, n = 5 x 4). Après euthanasie de 10 animaux contrôles (5 du groupe A et 5 du groupe B) et de 10 animaux traités par la CAP par inhalation d'éther à J6, J9, J12 ou J15, l'existence de processus apoptotiques a été recherchée dans les follicules ovariens par la méthode TUNEL ainsi que par la mise en évidence éventuelle d'une expression accrue de caspase 3 sous forme active par immunohistochimie et par Western blot tandis que l'intensité de l'expression de Ki67 mesurée par immunohistochimie a permis d'évaluer les phénomènes de prolifération cellulaire. L'atrésie folliculaire a été observée dans les follicules antraux de types D et E sur toutes les sections ovariennes mais, dans le groupe traité par la CAP, les index apoptotiques associés à l'atrésie ont été significativement plus faibles que ceux mesurés chez les contrôles particulièrement à J9 et J15 et l'accumulation intracytoplasmique de la caspase 3 active a également été significativement effondrée. En revanche, les index de prolifération cellulaire au sein des follicules ont été considérablement augmentés à J12 et J15 chez les rongeurs traités. Ces résultats indiquent que l'administration répétée de faibles doses de CAP interfère avec le développement des follicules ovariens en inhibant partiellement l'apoptose liée à l'atrésie et en promouvant la prolifération folliculaire, probablement par libération de certains neuropeptides présents dans les neurones sensoriels sensibles à la CAP.

Mots clés : Capsaïcine, follicules ovariens, rat, pré-puberté, apoptose, prolifération cellulaire, TUNEL, caspase 3, Ki 67.

Introduction

Endocrine [29], immune [24], paracrine and autocrine factors [25] as well as nervous system [30] are involved in the regulation of ovarian folliculogenesis and atresia. Various sympathetic, cholinergic, peptidergic and sensory nerve fibbers are in contact with ovaries and it is generally admitted that both the sympathetic and sensory extrinsic nerves are associated with the control of growing follicles, interstitial tissue, and ovarian vasculature [2, 18]. Sensory innervations play a role in the regulation of ovarian function, particularly for responses to gonadotropins [20].

Capsaicin (CAP) (8-methyl-N-vanillyl-6-nonenamide), the main ingredient of hot chilli peppers, selectively activates the peripheral terminal of sensory C and Aδ fibbers, generally referred as polymodal nociceptors [31]. It is currently being utilized for therapeutic treatment of various peripheral conditions such as rheumatoid arthritis and diabetic neuropathy [31]. These therapeutic effects of capsaicin are believed to be due to the modification of sensory nerve endings. Vanilloid receptor subtype 1 (VR1), a receptor responsible for capsaicin action, has been cloned by CATERINA *et al*. [5] from rat dorsal root ganglia and is considered as a common molecular target for protons and noxious heat (> 43°C) as well as for vanilloid compounds. The effects of CAP are dependent on the concentration of capsaicin and of the mode of application. Capsaicin at a low dose stimulates release of neuropeptides

such as catecholamines, neurokinin A (NKA), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and substance P (SP) from sensory neurons endings [12, 32]. In contrast, high dose capsaicin shows neurotoxic effect and induces an irreversible long-standing inactivation of the capsaicin-sensitive nerve endings with a loss of their sensory-afferent functions and their ability to release sensory neuropeptides [13]. Because of neurotoxic properties, high dose capsaicin is widely used as a specific tool to examine the function of sensory neurons. The effects of high doses of capsaicin are well-established on the ovary. Sensory nerves innervating the ovary have been shown to contain SP [8, 23], CGRP and VIP [4, 16]. SP fibbers predominate around the ovarian blood vessels, but it also has been noted that some fibbers are involved in modulating different stages of follicle development [15]. Several studies have demonstrated that in female reproductive organs, SP-immunoreactivity, NKA, CGRP, galanin, VIP, and somatostatin were markedly decreased following high dose capsaicin injection to neonatal rats [7, 12]. Also, neonatal capsaicin treatment destroyed the SP containing primary afferent nerves innervating the female rat reproductive tract and resulted in marked infertility in subsequent adult life [33]. In a recent publication, PINTADO *et al*. [28] reported that the neonatal treatment with high doses of capsaicin in female rats and mice resulted in a decreased reproductive success as compared with control rats. These researchers have suggested that capsaicin-sensitive sensory nerves could play a role in regulating of the fertility and follicle development in females [28, 33].

Capsaicin is not only used as a research tool or topical ointments for the treatment of pain but also is consumed regularly by many people as hot chilli pepper. The amount varies between the ethnical groups but most people are exposed to low levels of capsaicin through diet. Although the effects of high doses of capsaicin are well-established on the ovary, the effect of repeated exposure to low doses of capsaicin is not known. In the present study, we investigated the effects of low dose of CAP on ovarian follicular growth and atresia in the prepubertal rats.

Materials and Methods

ANIMALS AND EXPERIMENTAL PROTOCOL

Eighty immature female Sprague-Dawley rats (21 day old rats) obtained from the Experimental Animals Breeding and Research Center, Uludag University, Turkey, were used throughout the experiments. The animals were housed five per cage in temperature (20-24 °C), humidity (60-70%), and lighting (12 h light/dark cycle) controlled conditions and were provided with feed and water *ad libitum*. The experimental protocols were approved by the Animal Care and Use Committee of the Uludag University and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Rats were randomly divided into 3 groups. The first group (control A, n = 5 x 4) remained without any treatment. The second group (experimental-CAP treated, n = 10 x 4) received daily subcutaneous injections of CAP (Sigma, St. Louis, MO, USA) prepared in a solvent consisting of 10% ethanol, 10% Tween 80, and 80% distilled water at the dosage of 0.5 mg/kg/d, and the third group (vehicle treated-control B, n = 5 x 4) received subcutaneously an equal volume of solvent every day. All animals were weighed daily. After 6, 9, 12 and 15 days, 10 CAP treated rodents, 5 controls A and 5 controls B were euthanized by ether inhalation for each time point.

One ovary from each rat was weighed and stored at -80 °C for western blot studies, while the other ovary was fixed in 10% neutral buffered formalin and processed routinely for immunohistochemical studies. After deparaffinisation and rehydration, sections were stained for cell proliferation (Ki 67) (NeoMarkers, Fremont, CA, USA) and active caspase 3 (Alexis, San Diego, CA, USA) by immunohistochemistry and in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labelling (TUNEL) was used to determine the apoptotic cells.

IMMUNOHISTOCHEMISTRY ANALYSIS

Standard streptavidin biotin peroxidase complex technique was carried out by using Histostain Plus Kit (Zymed, South San Francisco, CA). Antigen retrieval was carried out by boiling sections in microwave oven at 750 W in sodium citrate buffer (1 M, pH 6.1) for Ki 67 staining, and in 0.1 M Tris-HCI buffer with 5% urea (pH 10.0) for active caspase 3 (3 x 5 min). After cooling, slides were rinsed with PBS and endogenous peroxidase activity was blocked by 10 min incubation at room temperature in 3% H_2O_2 solution in distilled water. After blocking with non-immune serum for 1 hour to reduce nonspecific antibody binding, sections were incubated with primary antibodies, a rabbit monoclonal antibody to Ki 67 (Clone SP6) diluted to 1:1000 or a rabbit polyclonal antibody to active (cleaved) caspase 3 (ALX-210-807) diluted to 1:50 for overnight at 4°C. Sections were then incubated with biotinylated secondary antibody for 10 min at room temperature followed by application of streptavidin conjugated to horseradish peroxidase for 10 min at room temperature. Finally, 3.3'-diaminobenzidine (DAB) was used for colour development and counterstaining was performed with haematoxylin. Slides processed without primary antibodies were included for each staining as negative control.

IN SITU LOCALIZATION OF APOPTOTIC CELLS

To visualize apoptotic cells, serial sections from each specimen were stained with TUNEL method using an ApopTag in situ apoptosis detection kit (Chemicon, CA, USA) according to the manufacturer's protocol. Briefly, the deparaffinised and hydrated sections were washed in PBS and treated with 20mg/L proteinase K for 20 min at 37°C. Specimens rinsed in PBS were immersed in 3% H_2O_2 for 5 min at room temperature to inhibit endogenous peroxidase activity. After rinsing in distilled water for 10 min, the equilibration buffer was applied to sections for 10 min and then they were incubated at 37°C with working buffer containing reaction buffer and terminal deoxynucleotidyl transferase (TdT) enzyme in a

humidified chamber for one hour. The reaction was stopped by soaking sections in wash buffer, followed by rinsing in PBS. The samples were later incubated in anti-digoxigenin peroxidase conjugate for 30 min at room temperature. After washing in PBS, sections were immersed for 10 min in DAB (3.3'-diaminobenzidine) for peroxidase colouring reaction. Sections were then counterstained with methyl green (0.5% in 0.1 M sodium acetate, pH 4.0), rinsed in distilled water, and examined under microscope (Nikon Eclipse 80i; Tokyo, Japan). As negative controls, sections were incubated with TUNEL label only by omitting either TdT or anti-digoxigenin antibody. Positive control sections received the same treatment but were pre-treated with DNase I (Roche, Indianapolis, USA) for 30 min at 37°C prior to TUNEL.

WESTERN BLOT ANALYSIS

Ovarian tissue were crushed under liquid nitrogen and lysed mechanically in lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholic acid, 0.1% SDS, 1% protease inhibitor cocktail). The ovary lysates were sonicated (three times for 10 sec on ice) in lysis buffer then sonicates were pelleted by centrifugation (14 000 g, 30 min, 4°C) and the supernatant fractions were carefully harvested and stored at -20°C. Protein concentrations in extracts were determined with the Bicinchoninic Acid Protein Assay Kit (Sigma). After mixing with sample buffer and boiling for 5 minutes, the proteins from the supernatant fractions were loaded and separated according to size by 15% SDS-PAGE for 60-80 min and then blotted onto polyvinylidene difluoride membranes (PVDF)(Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. Membranes were washed three times with TBS-T (0.5% Tween 20 in TBS buffer) for 10 min, blocked with 5% non-fat dry milk in TBS-T for 1 hour at room temperature to reduce nonspecific binding. After incubation for 2 hours at room temperature with rabbit anti-cleaved caspase 3 antibody (1:25), membranes were washed in TBS-T and incubated again for 1 hour at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (1:2000) (Santa Cruz Biotechnology, sc: 2030) and finally washed again in TBS-T twice. Peroxidase activity was visualized using chemiluminescent peroxidase substrate (Santa Cruz Biotechnology, sc-2048). The cleaved caspase 3 content was exposed to x-ray film. Relative band intensities were determined by quantification of each band with Image J Analyzer (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/).

QUANTITATIVE EVALUATION OF IMMUNOSTAINING AND TUNEL

Firstly, follicles were classified into the general categories listed in Table I according to Pedersen and PETERS [27] and BYSKOV [3].

Intensity of immunostaining and TUNEL was rated independently by two observers after the examination of all follicles at the largest section under direct visualization at X400 magnification. The apoptotic index was determined with evaluation of TUNEL stained sections on a scale between 0-2, where a follicle that contained 5% or less labelled granulosa cells was scored as 0. Follicles with 5-10% labelled granulosa cells received a score of 1, and follicles with more than 10% labelled granulosa cells were scored as 2 and were classified as atretic. The percentage of atretic follicles was defined as the ratio of atretic follicles to the total follicle number [10]. Active caspase 3 immunostaining was evaluated according to the staining intensity as follows: -: no staining, 1+: slight, 2+: moderate and 3+: strong [34].

The proliferation index (PI) was determined with Ki 67 score evaluation on a 2-point scale. A follicle that contained less than 10% labelled granulosa cells was scored 0; follicles containing 10-50% labelled granulosa cells was scored 1, and follicles with a majority of labelled granulosa cells received a score of 2. Follicles scored with 1 or 2 were classified as proliferating follicles. The percentage of proliferating follicles was defined as the ratio of proliferative follicles to the total follicle number [10].

STATISTICAL ANALYSIS

Statistical significance between the groups was analyzed by one way ANOVA test followed by Dunn's post-hoc test. The level of significance was defined as follows: a, b different from control A at $P \leq 0.05$ and $P \leq 0.001$ respectively; c, d, e different from control B at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively $P \leq 0.001$.

Class	Name	Description
A	Primordial	A single layer of squamous granulosa cells
B	Primary	Less than two layers of cuboidal granulosa cells with no thecal layer
C	Secondary	Three to six layers of cuboidal granulosa cells
D	Small antral	≤5 layers of cuboidal granulosa cells; definitive theca layer; antral cavities began to form
E	Large antral	Layers of cuboidal granulosa cells with columnar rearrangements at border of basement membrane
F	Graff	Mature Graffian follicles with well developed granulosa, thecal, and antral elements, cumulus-oocyte complex

TABLE I: Ovarian follicle classification.

Results

All stages of follicles except Graaf follicles (F, Table I) were observed in ovarian sections from the 3 groups of prepubertal rats.

Apoptosis was assessed by immunohistochemistry for active caspase-3 protein and *in situ* TUNEL method. While no TUNEL staining was detected in control sections processed without TdT enzyme, staining was observed in nuclei of all cells in positive control sections pre-treated with DNase and strong positive TUNEL reactions were evidenced in granulosa cells (more than 10% of labelled cells) from D and E type follicles (figure 1) which were morphologically considered as atretic. In parallel, no active caspase 3 immunoreactivity was observed in control sections but marked positive reactions (score: 3+) were detected in the cytoplasm of interstitial cells localized among the follicles, granulosa cells and of some theca follicle cells from atretic follicles (types D and E). However, some rare positive cells for TUNEL reaction and for active caspase 3 immunostaining were also encountered in some developmental follicles.

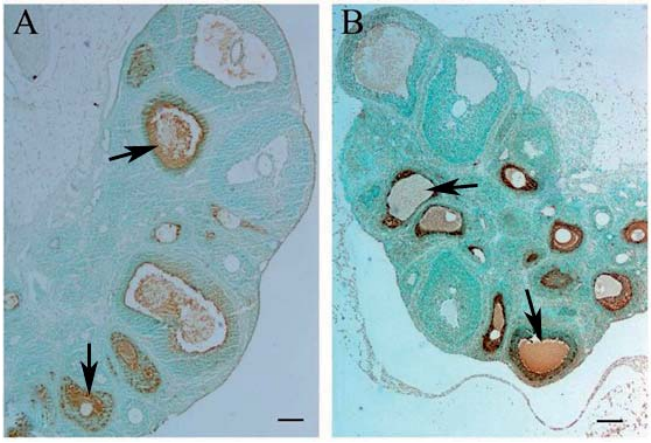

FIGURE 1 : Positive TUNEL reactions in atretic follicles (arrows) from prepubertal rats after 15 days of treatment with CAP (low dosage: 0.5 mg/kg/d, S.C.) (**A**) or with vehicle only (control B) (**B**). Bar 100 µm.

As expected, atretic follicles were observed in all groups. During the 15 days long experimental period, the apoptotic index greatly fluctuated in A and B controls, was minimal at the 9th day and maximal at the 15th day (figure 2). Although these fluctuations were also recorded in the CAP group, the apoptotic index was deeply decreased in atretic follicles type D and E at a lesser extend from treated rats: markedly and significantly depressed apoptotic indexes compared to controls were found on day 9 (vs. controls A: $P < 0.001$ for types D and E; vs. controls B: $P < 0.01$ for type D and $P < 0.05$ for type E) and this parameter remained significantly lower in treated rats than in controls A ($P < 0.001$ for type D and $P < 0.05$ for type E) and controls B ($P < 0.001$ for type D) on day 15.

Active caspase 3 protein expression was also determined in ovary by western blot analysis which revealed a single band at 13 kDa in ovary extracts and confirmed the significantly lower expression of the enzyme in the CAP treated rats compared to controls A and B ($P < 0.001$) (figure 3) as demonstrated with immunohistochemistry.

Ovarian cell proliferation was assessed by immune detection of the Ki67 antigen. Ki67 immunoreactivity was mainly localized in the nuclei of granulosa cells in the secondary follicles (C), small antral follicles (D) and large antral follicles (E) in all groups (figure 4). In the CAP treated group, some granulosa cells and oocytes of few primary and primordial follicles were also stained. No staining was observed in the interstitial tissue, atretic follicles, and in sections processed without primary antibody.

The proportion of proliferating follicles was higher in control A group than in both control B and CAP treated animals on days 6 and 9 (figures 5A and 5B), the differences being statistically significant for follicles of the type C ($P < 0.05$) and of the type D ($P < 0.001$) on day 9, whereas an opposite situation was encountered on days 12 and 15 (figure 4): on day 12, the proliferation indexes in follicles C, D and E have greatly decreased in both control groups and appeared similar except in follicles E where this parameter was significantly

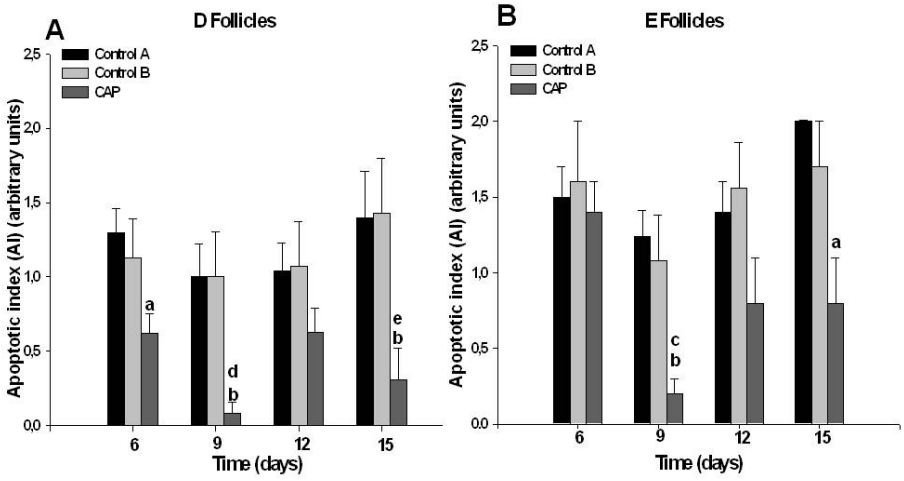

FIGURE 2: Apoptotic index (A.I.) for D (**A**) and E ovarian follicles (**B**) from prepubertal rats without any treatment (control A), after 15 days of treatment with CAP (low dosage: 0.5 mg/kg/d, S.C.) (group CAP) or with vehicle only (control B).
Superscripts *a,b* indicate significant differences ($P \leq 0.05$ and $P \leq 0.001$, respectively) with control A; Superscripts c,d,e indicate significant differences ($P \leq 0.001$) with control B.

higher in controls B than in controls A ($P < 0.05$). But, at this date, the proliferation intensity measured in the CAP group was dramatically increased compared to the controls A ($P < 0.05$) for the follicles E, and compared to the both 2 control groups for follicles C and D ($P < 0.001$) (figure 5C). On day 15 (figure 5D), the differences between CAP treated animals and controls were exacerbated and whatever the follicle C, D and E stages, higher proliferation indexes were observed in treated rodents ($P < 0.001$).

Discussion

CAP affects many systems in the organism, including nervous, cardiovascular, respiratory, immune, and gastrointestinal systems [1, 14, 19]. Little is known about the effects of CAP on the female reproductive system. Many researchers used high doses of CAP as a specific tool to examine the function of sensory neurons in the reproductive system [20, 21].

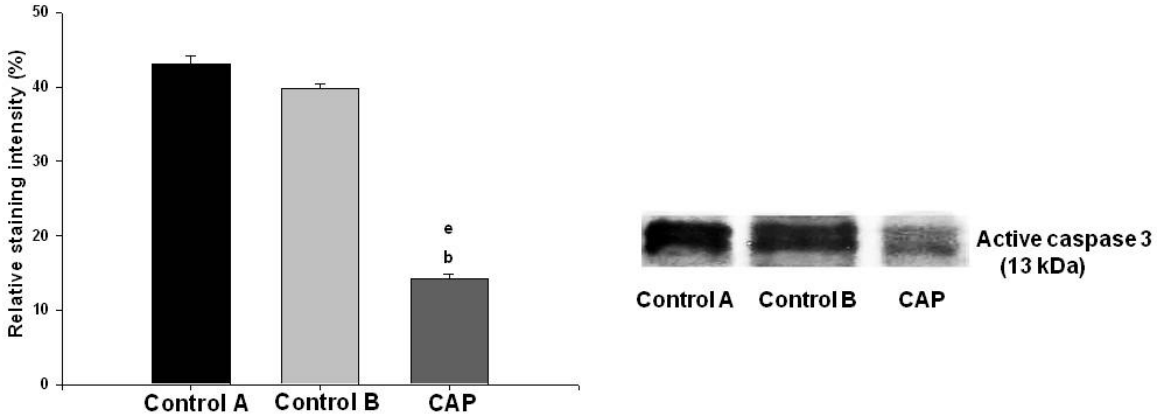

FIGURE 3: Western blot intensity of the active caspase 3 (band: 13 kDa) extracted on Day 15 from ovaries of prepubertal rats without any treatment (control A), after 15 days of treatment with CAP (low dosage: 0.5 mg/kg/d, S.C.) (group CAP) or with vehicle only (control B). Results are expressed as mean ± standard errors.
Superscripts b,e indicate significant differences ($P \leq 0.001$) with controls A and B respectively.

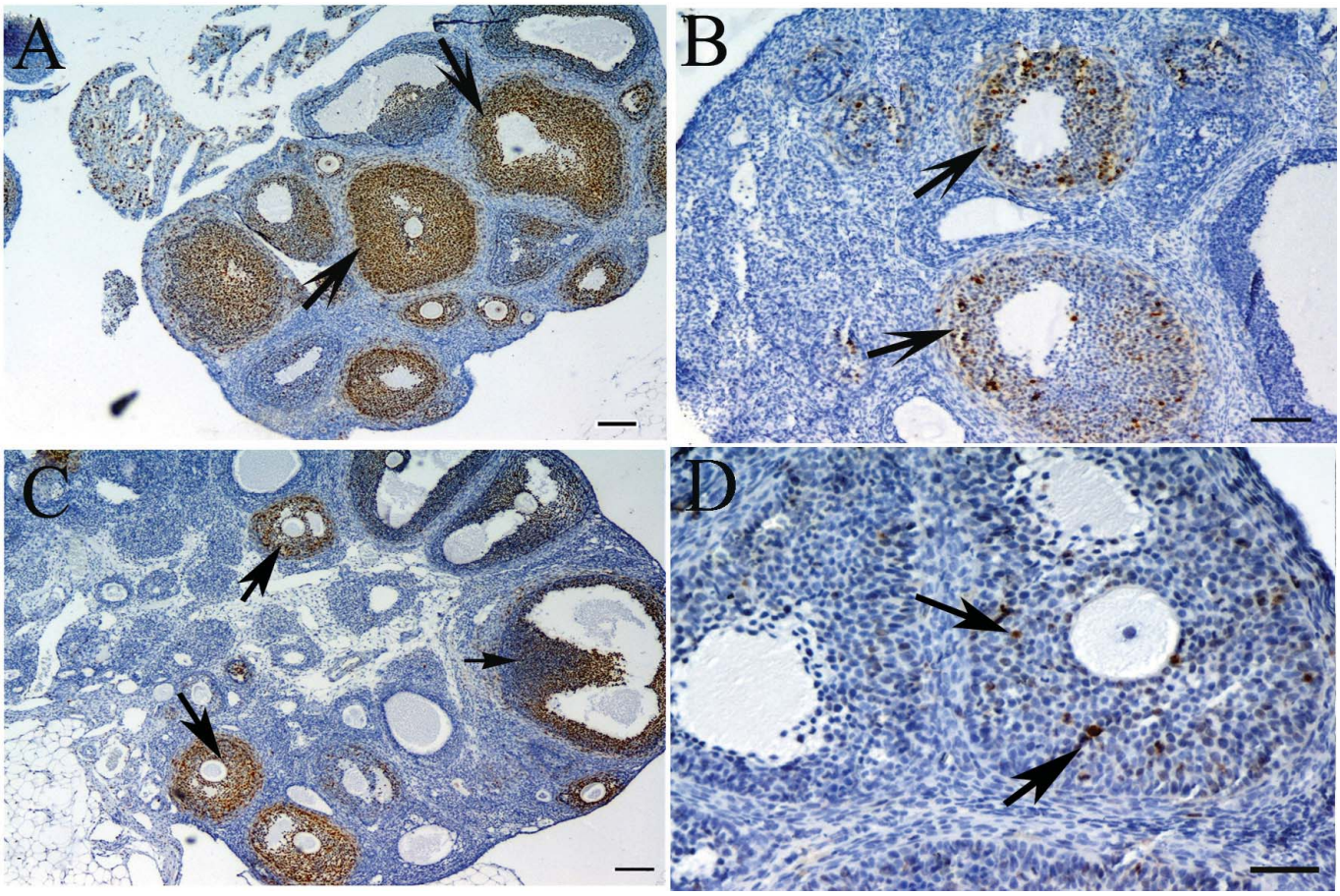

FIGURE 4: Ki67 immunostaining showing proliferating follicles (arrows) in ovaries from prepubertal rats treated with CAP (low dosage: 0.5 mg/kg/d, S.C.) (**A** and **C**, Bar 100 μm) or not treated (control A) (**B** and **D**, Bar 50 μm) collected on Day 12 (**A** and **B**) or on day 15 (**C** and **D**).

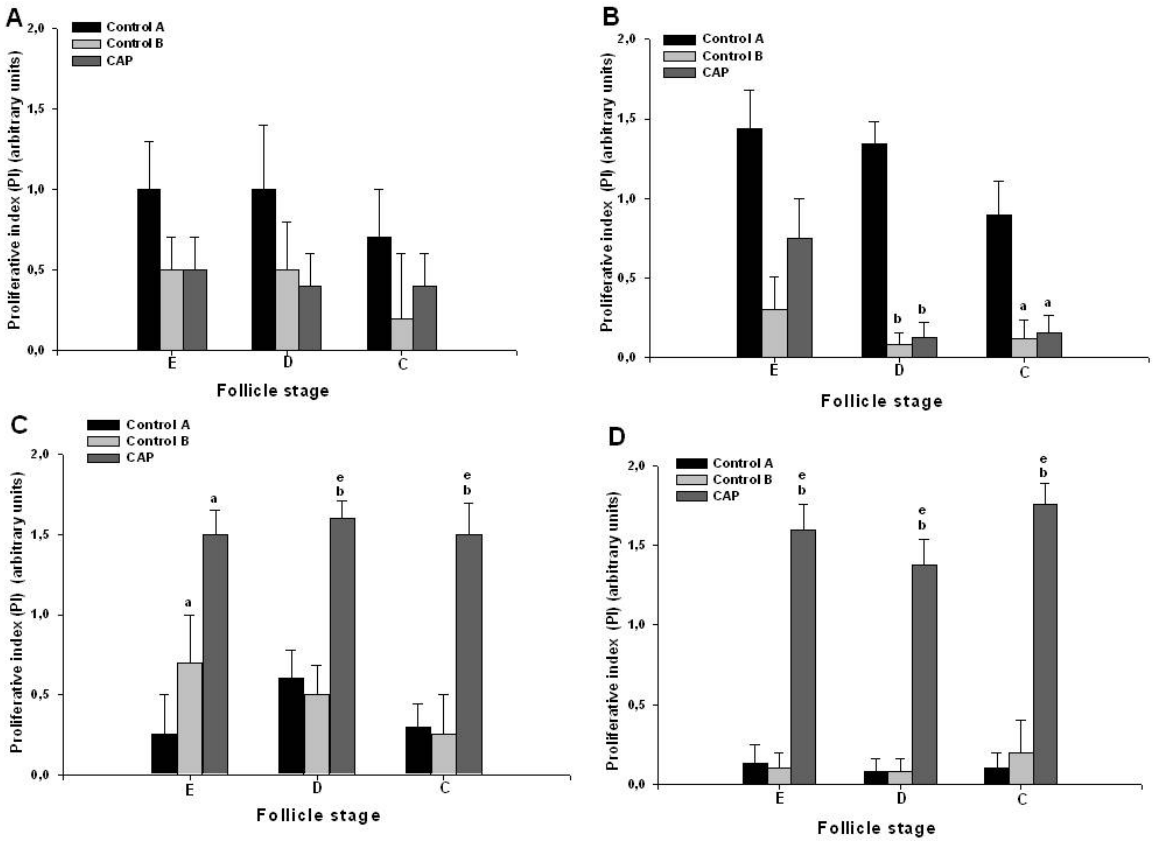

FIGURE 5: Proliferative index (P.I.) for C, D and E ovarian follicles from prepubertal rats without any treatment (control A), treated with CAP (low dosage: 0.5 mg/kg/d, S.C.) (group CAP) or with vehicle only (control B) determined for 6 (**A**), 9 (**B**), 12 (**C**) or 15 days (**D**) of treatment.

Superscripts a,b indicate significant differences ($P \leq 0.05$ and $P \leq 0.001$, respectively) with control A; Superscript e indicate significant differences ($P \leq 0.001$) with control B.

MORAN *et al*. [20] investigated the effects of high dose CAP (50mg/kg) on 3 days old denervated female rats' reproductive system. These researchers determined that CAP treated animals' follicles and ova shed numbers were significantly lower than control groups and they observed an increase of the number of atretic follicles compared to control groups. Although the effects of high doses of capsaicin are well-established on the ovary, the effect of repeated exposure to low doses of capsaicin is not known. This is the first study about effects of low CAP dosage on rat ovary. In this study, TUNEL positivity and the active caspase 3 expression are together assessed to determine atretic follicles, as atresia is associated with apoptosis [22].

Consistent with this, apoptosis was evidenced by TUNEL and active caspase 3 immunohistochemistry both in the theca cells and granulosa cells of atretic follicles. In this study, atresia was only observed in the antral follicles (types D and E) and not in other follicles. In accordance with our findings, several studies showed that in the healthy human and mouse ovaries, the follicular atresia was determined only in the antral follicles but not detected in the pre-antral follicles [9, 17]. In rat ovary, follicular development and atresia are at peak during pre-puberty [11]. Large antral follicles that escape from atresia become dominant follicles and are chosen for ovulation [6]. Our results showed that the follicular atresia was significantly lower in the CAP treated groups than in other groups, suggesting that CAP may prevent atresia formation. Consequently, the low level of follicular atresia in antral follicles induced by CAP treatment may be associated with higher numbers of ovulating follicles and higher fertility rate in these animals.

On the other hand, the ovarian cell proliferation was assessed by immuno-detection of the Ki 67 antigen. As expected, Ki67 immunoreactivity negatively correlated with TUNEL and the active caspase 3 expression, and the Ki67 expression was observed in the granulosa cells of type C, D and E follicles of all groups. Remarkably, on days 12 and 15, the proliferative indexes measured in ovarian developmental follicles from the CAP treated rodents were significantly higher than that of control groups. These results suggest that low dose of CAP inhibits apoptosis and stimulates the proliferation of granulosa cells.

Previously, we studied the effects of red hot pepper in the reproductive system of laying hens fed with a diet containing red hot pepper [26] and demonstrated that red hot pepper stimulated the follicular development and improved laying performance. Neuropeptides in CAP sensitive sensory neurons, such as CGRP, cholecystokinin, nitric oxide and SP may mediate the observed effects of CAP [12, 28, 32]. It has been demonstrated that these peptides may increase the blood

flow to the gonad and be involved in the regulation of female reproductive function [8, 15]. It remains to be determined, however, whether the observed anti-apoptotic and pro-proliferative effects seen with CAP in the current study was due to the release of one or several specific neuropeptides.

As a conclusion, it would be postulated that low dose of CAP protects the follicles from apoptosis and atresia, and stimulates follicular development.

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