Distribution of the vanilloid (capsaicin) receptor type 1 in the capsaicin treated rat ovaries on different sexual development periods

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SUMMARY

The intra-ovarian expression of the vanilloid receptor type 1 (VR1) was investigated in the present study on rats chronically treated with low dose capsaicin (CAP) according to the sexual development periods. Ovaries were removed at the puberty (42 day old), at the postpuberty (56 day old) or in adult stage (70 day old) from female Sprague Dawley rats receiving daily subcutaneous injections of capsaicin (1 mg/kg/d) diluted into 10% ethanol, 10% Tween 80, 80% distilled water (CAP group), or treated only with the vehicle (VEH group) or not treated (C group) (n = 10 in each subgroup) and tissue samples were processed for conventional histology and for immunohistochemistry using the Streptavidin-Biotin Peroxidase method and a rabbit polyclonal anti-VR1 primary antibody. Serum gonadotropin and sexual steroid concentrations were determined in parallel by commercial ELISA kits. Whatever the sexual development state, the VR1 expression was evidenced in the cytoplasm of various ovarian cell types such as the granulosa, interstitial and luteal cells as well as theca cells at a lesser extend and appeared independent from a hormonal control and from capsaicin treatment. On the other hand, the number of developing follicles has increased in ovaries from the capsaicin treated rats whereas the FSH and LH secretion appeared to be constantly strengthened during the sexual development periods and the ovarian sexual steroid production was globally markedly decreased. Nevertheless, the vehicle injections have also interfered with the hormonal control with highly sustaining the progesterone production. These results showed the constitutive expression of the VR1 in rat ovaries and the positive effects of a chronic capsaicin treatment on the proliferating phases of the follicle development but further studies are required for identifying the specific actions of the drug.

Keywords: Rat, ovary, capsaicin, vanilloid receptor, immunohistochemistry, sexual development, gonadotropins, sexual steroids.

RÉSUMÉ

Distribution intra-ovarienne du récepteur de type 1 de la vanilloïde (capsaïcine) en fonction du stade de développement sexuel chez les rattes traitées par la capsaïcine

Dans cette étude, l'expression intra-ovarienne du récepteur de type 1 à la vanilloïde (VR1) a été recherchée chez des rattes traitées chroniquement par une faible dose de capsaïcine (CAP) en fonction du stade de développement sexuel. Au moment de la puberté (âge: 42 jours), à la post-puberté (âge : 56 jours) et à l'âge adulte (70 jours), les ovaires ont été prélevés sur des femelles Sprague Dawley ayant reçu des injections sous-cutanées quotidiennes de capsaïcine (1 mg/kg/j) diluée dans 10 % éthanol, 10 % Tween 80 et 80 % d'eau distillée (groupe CAP), ou de solvant uniquement (groupe VEH) ou n'ayant reçu aucun traitement (groupe C) (10 animaux par sous-groupe) puis les tissus ont été classiquement analysés par histologie conventionnelle et par immunohistochimie en utilisant la méthode de marquage par la streptavidine / biotine / peroxydase et un anticorps polyclonal de lapin anti-VR1. Les concentrations sériques des gonadotrophines et des stéroïdes sexuels ont été mesurées à l'aide de kits ELISA spécifiques. Quelle que soit la période de développement sexuel, l'expression du VR1 a été mise en évidence dans le cytoplasme de différentes cellules ovariennes (cellules de la granulosa, interstitielles, du corps jaune et de la thèque à un moindre degré) et est apparue indépendante d'une quelconque stimulation hormonale ainsi que du traitement par la capsaïcine. Par ailleurs, le nombre de follicules en cours de développement a augmenté dans les ovaires des rattes traitées par la capsaïcine alors que les sécrétions de LH et de FSH sont apparues renforcées mais stables au cours du développement sexuel et que la production ovarienne des stéroïdes a été globalement et nettement diminuée. Néanmoins, les injections de solvant ont aussi affecté les productions hormonales en maintenant, en particulier, une progestéronémie élevée. Ces résultats démontrent une expression ovarienne constitutive du VR1 chez le rat ainsi que les effets positifs d'un traitement chronique par la capsaïcine sur les phases de prolifération au cours du développement folliculaire mais des études ultérieures sont à mener pour identifier avec précision les actions spécifiques de cette molécule.

Mots clés : Rat, ovaire, capsaïcine, récepteur de la vanilloïde, immunohistochimie, développement sexuel, gonadotrophines, stéroïdes sexuels.

Introduction

Ovarian folliculogenesis and atresia is regulated by interactions between endocrine [38], immune [33], paracrine-autocrine factors [34] and the nervous system [39]. It is generally well admitted that both the sympathetic and the sensory extrinsic nerves are associated with the control of growing follicles, interstitial tissue, and ovarian vasculature [4, 11, 26]. In rats, ovaries receive neural information through sympathetic, cholinergic, peptidergic and sensory nerve fibres. Sensory innervations play a role in the regulation of ovarian function and the afferent sensory nerves are involved in the regulation of ovarian folliculogenesis or atresia in response to gonadotropins [29]. Sensory nerves innervating the ovary have been shown to contain SP (substance P) [8, 32, 35], CGRP (calcitonin gene-releated peptid) and VIP (vasoactive intestinal polypeptide) [5, 20]. It has been proposed that ovarian innervations register functional information via receptors localized around the follicles [9]. Several studies have demonstrated that in fe-

male reproductive organs, SP-immunoreactivity, neurokinin A, CGRP, galanin, VIP and somatostatin show a marked decrease following capsaicin injection to the neonatal rats [7, 12, 44]. A subpopulation of primary sensory neurons is stimulated and subsequently desensitized by capsaicin. In general, these neurons are peptidergic, with a small to a medium diameter and constitute unmyelinised C fibres or thinly myelinised Aδ fibres [42]. The function of capsaicin is mediated via the stimulation of a specific membrane-bound vallinoid receptor. This receptor has been localized on primary afferent neurons in cervical, thoracic, and lumbar segments [50] and a constitutive VR1 expression in rat ovaries was recently demonstrated [45].

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide, CAP) is the major pungent ingredient of hot peppers [12]. It excites sensory neurons that have unmyelinised or thinly myelinised nerve fibres and produces a sensation of burning pain or irritation and activates protective reflexes and autonomic responses in experimental animals and in humans [42]. Moreover, capsaicin is known to release and deplete the SP [16], the neurokinin A [37] and the CGRP from sensory nerves, thereby eliciting neurogenic inflammation at the site of stimulation [22]. The effects of capsaicin 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, VIP, CGRP and SP from sensory neurons endings [12, 41, 43]. In contrast, capsaicin at high dose 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]. The effects of high doses of capsaicin are well-established on the ovary. SP fibres predominate around the ovarian blood vessels, and it has also been noted that some fibres are involved in modulating different stages of the follicle development [18]. Several studies have demonstrated that in female reproductive organs, SP-immunoreactivity, neurokinin A, CGRP, galanin, VIP, and somatostatin, show a marked decrease following high dose capsaicin injections to neonatal rats [7, 12]. Additionally, 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 [44]. In a recent publication, PINTADO et al. [36] reported that the neonatal treatment with capsaicin at high doses in female rats and mice resulted in a decreased reproductive success compared to control rats. These researchers have suggested that capsaicin-sensitive sensory nerves could play a role in regulating the fertility and follicle development in females [36, 44].

There is a large body of evidence indicating that the excitatory effect of capsaicin on sensory neurons is due to its ability to increase the open state of a channel previously defined as the 'capsaicin receptor'. The recent cloning of this molecular entity has revealed that it consists in a 426 amino-acid protein, which has been firstly termed vanilloid receptor-1 (VR1) [6]. The vanilloid receptor [transient receptor potential (TRP) V1, also known as VR1] is a member of the mammalian transient receptor potential (TRP) channel family [47]. Mammalian TRP channels are regrouped into six related protein subfamilies known as TRPV, TRPC, TRPM, TRPP, TRPN and TRPA. The TRPV1 channel a member of the TRPV subfamily, is referred as the vanilloid receptor 1 (VR1) or the capsaicin receptor

[30]. VR1 is mainly expressed in primary afferent neurons and this receptor is responsible for the capsaicin action. It has been cloned by CATERINA et al. [6] from rat dorsal root ganglia and was recognized as a common molecular target for protons and noxious heat ($> 43°C$) as well as for vanilloid compounds [22]. VR1 is highly expressed in a subset of primary sensory neurons but it is also expressed in diverse areas of the central nervous system including the limbic system, striatum, hypothalamus, hippocampus, and cerebellum [27]. Although the role of the VR1 in the central nervous system is still elusive, it may mediate endovanilloid signalling by promoting the release of excitatory neurotransmitter [23, 24]. There is also evidence that mRNA and protein of VR1 are produced and expressed in non-neuronal cells, including the urinary bladder epithelial cells [2], keratinocytes [14], mast cells [40], human bronchial epithelial cells and rat gastric epithelial cells [17].

However, the VR1 expression and its functionality are not well elucidated in the ovaries. The aim of the present investigation was to determine the localisation of VR1 in rat ovaries by immunohistochemistry and to identify possible differences in the ovary structures for VR1 immunoreactivity during developmental periods after application of capsaicin at a low dose for a prolonged period in the rat ovaries.

Materials and Methods

ANIMALS AND EXPERIMENTAL PROTOCOL

The rats were obtained from the Experimental Animals Breeding and Research Centre, Uludag University, Turkey. Ninety immature female Sprague-Dawley rats, 21 day old at the beginning of the experiment, were used. The animals were handled according to the approved national guidelines for animal care. They were kept under controlled light conditions (Light / Dark: 12h / 12h), in temperature (20-24ºC), humidity (60-70%) and fed with an ordinary laboratory diet. Experimental procedures were approved by the Uludag University Ethical Committee for animal experimentation (Protocol Number: 25.04.2006/1).

The rats were randomly divided into 3 equal groups (n = 30 rats in each group). Whereas the first group remained without any treatment (C group), the second group (experimental-capsaicin treated group or CAP group) received daily subcutaneous injections of 1 mg/kg/day capsaicin (Sigma, St. Louis, MO, USA), prepared in a solvent consisting of 10% ethanol, 10% Tween 80, and 80% distilled water, and the third group (vehicle treated group or VEH group) received daily subcutaneous injections of the equal volume of the solvent. All groups were divided into three subgroups (n = 10 in each subgroup) according to the stage of the sexual development: puberty at 42 day old, postpuberty at 56 day old and adult stage at 70 day old).

All animals were weighed daily. After euthanasia by ether inhalation and decapitation of all animals, ovaries were surgically removed and dissected from surrounding tissues. Tissues were fixed in 10% formaldehyde solution, and then tissue samples were embedded in paraffin blocks according to routine histological procedures. Sections (5μm thick) were stained

with Crossman's triple stain for the histological structure or immunostained for VR1 localisation.

IMMUNOHISTOCHEMISTRY

Polyclonal rabbit VR1 primary antibody (R-130, Santa Cruz) raised against amino acids 1-130 mapping at the N-terminus of rat VR1 was used for the VR1 immunostaining. The standard Streptavidin Biotin Peroxidase complex technique was carried out using the Histostain Plus Kit (Zymed). Briefly, the sections were deparaffinised, hydrated and processed for antigen retrieval using microwave oven. The sections were washed with buffered citrate (pH 6) for 5x2 minutes. Endogenous peroxidase activity was blocked after 10 minutes incubation in 3% H_2O_2 solution in distilled water. Non-specific antibody binding was reduced by the tissue incubation in non-immune serum blocking solution for 1 hour at room temperature before application of the VR1 antibody. The sections were incubated overnight at 4ºC with the anti-VR1 primary antibody diluted at 1:1000. The sections were incubated with biotinylated secondary rabbit antibody for 10 minutes followed by streptavidin conjugated to horseradish peroxidase for 10 minutes at room temperature. Finally, 3.3'-diaminobenzidine (DAB) was used as enzyme substrate and colour development and haematoxylin was used for counterstaining. Negative control slides processed without primary antibodies were included for each staining. All the slides were coded in order to assure a blind analysis and graded according to the following scale: - no staining (0%), + slight (10-25%), ++ medium (25-50%), +++ strong (50-100%) [1].

SERUM HORMONE CONCENTRATIONS

Before decapitation blood was collected from heart in all rats in sterile tubes without anticoagulant. After clotting for 1 hour at room temperature, blood samples were centrifuged at 3 000 g for 5 minutes at room temperature and sera were carefully harvested and stored at –20°C until assayed. Serum LH, FSH, oestrogen, progesterone concentrations were measured by Enzyme-Linked Immunosorbent Assay kits (ELISA, Endocrine Technologies, Inc.).

STATISTICAL ANALYSIS

Statistical differences between groups were analyzed by one way ANOVA test followed by Dunn's post-hoc test (Sigma Stat 3.1). Differences were considered as significant when P values were less than 0.05.

Results

No mortality case in any group was recorded during the whole experimental period. As shown in the Table I, during the puberty and postpuberty periods, the mean body weight gains were higher in the not treated control rats than in the 2 other groups, injected with the capsaicin (CAP group: $P < 0.05$ for the postpuberty period) or with the vehicle alone (VEH group: $P < 0.05$ for the puberty period). In the adult rats, whereas the mean body weight gains were similar in the 2 control groups (not treated and treated with the vehicle alone), this parameter was markedly depressed in capsaicin treated animals ($P < 0.001$).

The mean ovary weights according to the sexual development periods were reported in the Table II. While the ovaries exhibited similar weights during the puberty period, they appeared bigger when rats were injected with capsaicin or with the vehicle alone (10% ethanol, 10% Tween 80, and 80% distilled water) compared to the not treated controls (C group) ($P < 0.05$). Moreover, maximal values were observed in the CAP group (CAP group vs. VEH group: $P < 0.05$ for the postpuberty period).

	Puberty (42 day old)	Postpuberty (56 day old)	Adult (70 day old)
C group	89.25 ± 2.93^a	132.30 ± 2.48^a	157.10 ± 2.31^a
VEH group	73.11 ± 3.51^b	123.50 ± 3.06^{ab}	158.20 ± 3.40^a
CAP group	80.60 ± 1.60^{ab}	118.70 ± 3.04^b	128.33 ± 3.12^b

Different superscripts ^{a,b} in the same column indicate significant differences between groups ($P < 0.05$).

TABLE I: Body weight gains (g) in rats treated with subcutaneously daily injection of capsaicin (1 mg/kg/day) diluted into 10% ethanol, 10% Tween 80, and 80% distilled water (CAP group) or with vehicle alone (VEH group) or without any injection (C group). Results are expressed as means ± standard errors.

	Puberty (42 day old)	Postpuberty (56 day old)	Adult (70 day old)
C group	28.0 ± 2.0	30.3 ± 2.0^a	38.7 ± 2.0^a
VEH group	29.9 ± 2.0	39.7 ± 4.0^b	46.8 ± 3.0^b
CAP group	25.6 ± 1.0	47.2 ± 3.0^c	52.5 ± 3.0^b

Different superscripts ^{a,b,c} in the same column indicate significant differences between groups ($P < 0.05$).

TABLE II: Ovary weights (mg) in rats treated with subcutaneously daily injection of capsaicin (1 mg/kg/day) diluted into 10% ethanol, 10% Tween 80, and 80% distilled water (CAP group) or with vehicle alone (VEH group) or without any injection (C group). Results are expressed as means ± standard errors.

The histological structures of ovaries according to the sexual development period were analysed by histology after Crossman's triple stain. The ovaries were seen covered by germinative epithelium and beneath this epithelium is the *tunica albuginea*. Blood vessels with various sizes and connective tissue cells were observed in the ovarian medulla. The ovarian cortex is composed of the connective tissue, ovarian follicles in various stages of development (primordial, primary, secondary, graffian and atretic follicles), interstitial cells and corpus luteum (figure 1). No corpus luteum was evidenced in ovaries during the puberty period and this histological follicle state has appeared during the postpuberty period. In adult rats, the number of developing follicles decreased while the number of corpus luteum was increased (figure 2a). However, a high density of primordial, primary and secondary follicles was observed in adult rats treated with capsaicin (figure 2b).

A VR1 immunopositive reaction was located in the cytoplasm of the granulosa cells, the interstitial cells, the theca and corpus luteal cells whatever the sexual developmental period of the female rats from the not treated control group (figure 3), from the vehicle-treated control group (figure 4) and from the capsaicin treated group (figure 5). No specific VR1 immunostaining was observed in the negative control slides (without the primary antibody). In the puberty period, the intensity of the VR1 immunolabelling was identically higher in the granulosa and interstitial cells from control ovaries than in the theca cells (figure 3a). By contrast, the interstitial cells have exhibited a lower immunostaining in the vehicle treated rats (figure 4a) whereas the immunolabelling of the granulosa cells was markedly increased in the capsaicin treated rats (figure 5a) (Table III). During the postpuberty period, the VR1 immunolabelling in the granulosa cells was remarkably intense in the 3 groups and higher than in the all other cell types. Interstitial cells and corpus luteal cells have similarly and moderately expressed the VR1 protein whereas the VR1 immunostaining remained low in the theca follicle cells (figures 3b, 4b and 5b, Table III). In adult not treated rats (figure 3c, Table III), the VR1 expression was similar in the granulosa cells and in the corpus luteal cells and remained strong compared to the in-

terstitial and theca cells. In vehicle and capsaicin treated adult rats (VEH and CAP groups) the VR1 expression pattern according to the ovarian cell type was identical (figures 4c and 5c, Table III): whereas the granulosa cells still exhibited a very intense VR1 labelling (more exacerbated than in the not treated control adults), the corpus luteal cells and the interstitial cells were equally and moderately immunostained and the VR1 expression in the theca cells remained low as in the not treated controls. However, no statistically significant difference in the VR1 immunostaining was evidenced according to the treatment or to the sexual development stage.

In parallel, the effects of the sexual developmental period and of the capsaicin treatment on the serum hormone FSH, LH, progesterone and oestrogen concentrations were investigated (Table IV). In the not treated controls, the LH concentrations remained stable according to the developmental state whereas the FSH concentrations dramatically varied, were very low in the puberty period, markedly peaked in the postpuberty period and abruptly declined in adults (postpuberty vs. puberty or adult stage: $P < 0.05$). The progesterone production tended to be constant in the puberty and postpuberty periods despite great value dispersion, but slightly declined in adults. The oestrogens exhibited similar variations according to the rat age but, nevertheless, they were markedly reduced in adults compared to the puberty or postpuberty periods ($P < 0.05$). The LH concentrations gradually increased according to the age in the vehicle treated rats (puberty vs. adult stage: $P < 0.05$) but were not significantly different from the control values for a given developmental stage and they appeared slightly elevated but not significantly in the capsaicin treated rats during the puberty and postpuberty periods compared to the controls. In the vehicle and capsaicin treated rats, the second pituitary hormone concentrations have also reached maximal values during the postpuberty period (postpuberty vs. puberty: $P < 0.05$) but the increase percentages of the FSH concentrations were lower than in the not treated controls (312%, 500% and 3617% for the CAP group, the VEH group and the C group, respectively) and the FSH concentrations remained increased in adults compared to controls ($P < 0.05$ for the VEH group).

	Granulosa cells	Interstitial cells	Theca follicle cells	Corpus luteal cells
Puberty				
C group (n = 10)	++	++	+	absent
VEH group (n = 10)	++	+	+	absent
CAP group (n = 10)	+++	++	+	absent
Postpuberty				
C group (n = 10)	+++	++	+	++
VEH group (n = 10)	+++	++	+	++
CAP group (n = 10)	+++	++	+	++
Adult				
C group (n = 10)	++	+	+	++
VEH group (n = 10)	+++	++	+	++
CAP group (n = 10)	+++	++	+	++

TABLE III: Intensity of VR1 immunostaining in ovary cells according to the sexual development (puberty at 42 day old, postpuberty period at 56 day old and adult stage at 70 day old) of rats treated with subcutaneously daily injections of capsaicin (1 mg/kg/day) diluted into 10% ethanol, 10% Tween 80, and 80% distilled water (CAP group) or with vehicle alone (VEH group) or without any injection (C group). Results are expressed semi-quantitatively: (-) no staining. (+) weak. (++) moderate. (+++) strong.
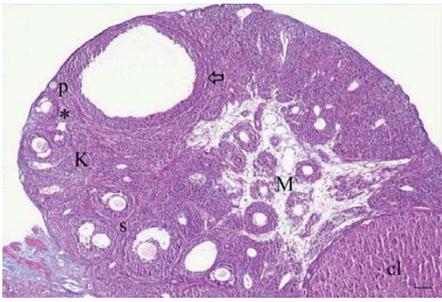
FIGURE 1: Histological ovary structure in a 56-days old (postpuberty period) not treated rat (C group): p: primordial follicle, *: primary follicle, s: secondary follicle, ⇨: atretic follicle, cl: corpus luteum, K: cortex and M: medulla. Crossman's triple stain, Bar: 100 μm.
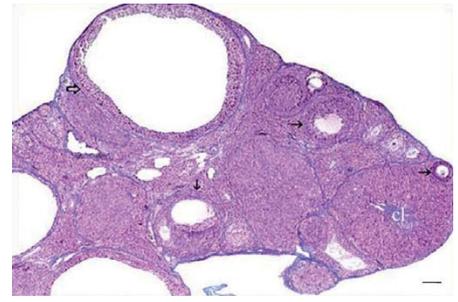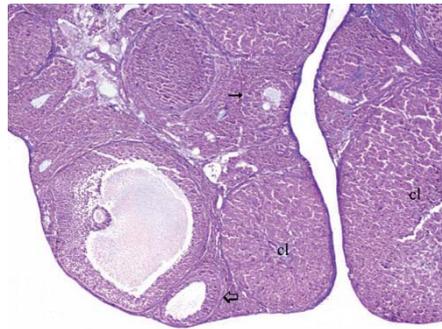
FIGURE 2: Histological ovary structures in adult rats (70 days old) from the not treated group (C group) (**a.**) and from capsaicin-treated group (CAP group) (**b.**): ↑: developing follicles, ⇨: atretic follicle, cl: corpus luteum. Crossman's triple stain, Bar: 100 μm.
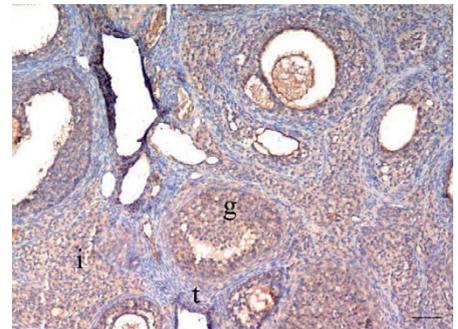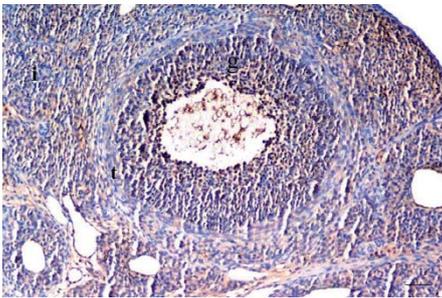

FIGURE 3: VR1 immunostaining in the ovarian cells from not treated control rats (C group) during the puberty period (42 day old rat) (**a.**), the postpuberty period (56 day old rat) (**b.**) and in adult rat (70 day old) (**c.**). g: granulosa cells, i: interstitial cells, t: theca follicle cells, cl: corpus luteal cells. Streptavidin Biotin Peroxidase complex technique using polyclonal rabbit VR1 primary antibody, Bar: 50 μm.
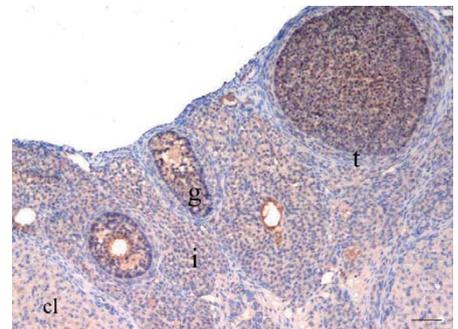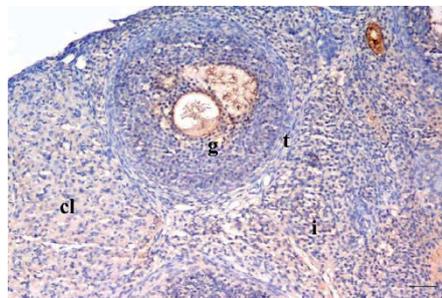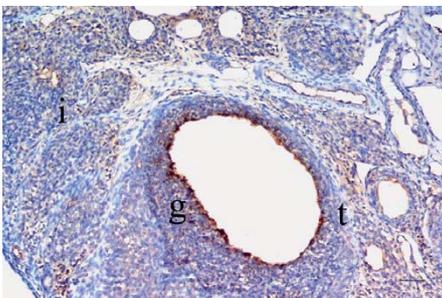

FIGURE 4: VR1 immunostaining in the ovarian cells from vehicle (10% ethanol, 10% Tween 80, and 80% distilled water) treated control rats (VEH group) during the puberty period (42 day old rat) (a.), the postpuberty period (56 day old rat) (b.) and in adult rat (70 day old) (c.). g: granulosa cells, i: interstitial cells, t: theca follicle cells, cl: corpus luteal cells. Streptavidin Biotin Peroxidase complex technique using polyclonal rabbit VR1 primary antibody, Bar: 50 μm.
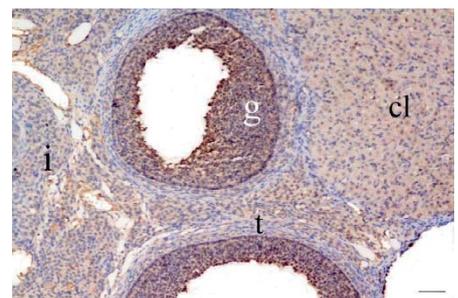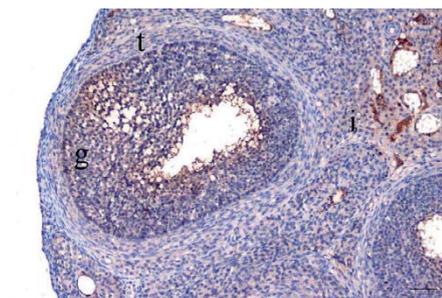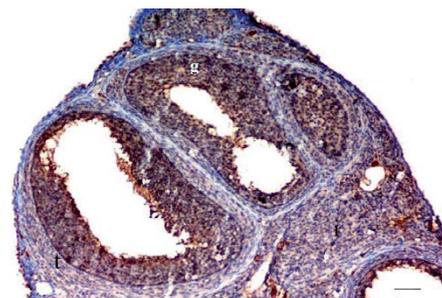
FIGURE 5: VR1 immunostaining in the ovarian cells from capsaicin treated rats (CAP group) during the puberty period (42 day old rat) (**a.**), the postpuberty period (56 day old rat) (**b.**) and in adult rat (70 day old) (**c.**). g: granulosa cells, i: interstitial cells, t: theca follicle cells, cl: corpus luteal cells. Streptavidin Biotin Peroxidase complex technique using polyclonal rabbit VR1 primary antibody, Bar: 50 μm.

	LH (µg/L)	FSH (µg/L)	Progesterone (µg/L)	Oestrogen (µg/L)
Puberty				
C group (n = 10)	0.479 ± 0.133	0.042 ± 0.300[aA]	2.181 ± 0.545[a]	626.712 ± 25.712[aA]
VEH group (n = 10)	0.373 ± 0.138[A]	0.140 ± 0.041[abA]	2.372 ± 0.402[aA]	133.374 ± 19.383[bA]
CAP group (n = 10)	0.634 ± 0.085	0.162 ± 0.053[bA]	1.091 ± 0.262[bA]	28.285 ± 0.000[cA]
Postpuberty				
C group (n = 10)	0.548 ± 0.098	1.561 ± 0.412[aB]	2.877 ± 1.216	634.310 ± 26.350[aA]
VEH group (n = 10)	0.533 ± 0.154[AB]	0.840 ± 0.310[abB]	4.012 ± 1.594[AB]	247.592 ± 37.484[bB]
CAP group (n = 10)	0.750 ± 0.251	0.668 ± 0.174[bB]	6.307 ± 1.882[B]	149.274 ± 19.368[cB]
Adult				
C group (n = 10)	0.447 ± 0.111	0.157 ± 0.060[aA]	1.436 ± 0.543[a]	231.880 ± 16.973[aB]
VEH group (n = 10)	0.654 ± 0.121[B]	0.587 ± 0.300[bAB]	3.961 ± 0.996[bB]	177.485 ± 19.082[bAB]
CAP group (n = 10)	0.464 ± 0.085	0.478 ± 0.159[abAB]	0.817 ± 0.104[cA]	125.076 ± 10.362[cAB]

Different superscripts [a,b,c] in the same column for a given sexual development stage (puberty, postpuberty or adult) indicate significant differences between groups (P < 0.05).

Different superscripts [A,B,C] in the same column for a given group indicate significant differences between the sexual development stages (puberty, postpuberty or adult) (P < 0.05).

TABLE IV: Serum hormone concentrations according to the sexual development (puberty at 42 day old, postpuberty period at 56 day old and adult stage at 70 day old) of rats treated with subcutaneously daily injections of capsaicin (1 mg/kg/day) diluted into 10% ethanol, 10% Tween 80, and 80% distilled water (CAP group) or with vehicle alone (VEH group) or without any injection (C group). Results are expressed as means ± standard errors.

Additionally, the pituitary hormone concentrations at the puberty period were significantly higher in the capsaicin treated rats than in the not treated controls (*P < 0.05*) whereas they were significantly lowered during the postpuberty (*P < 0.05*). The progesterone concentrations were similar in the vehicle treated and not treated groups at the puberty whereas they were significantly depressed by the capsaicin treatment (*P < 0.05*). Contrary to the not treated controls, the progesterone concentrations increased in the 2 following periods in the vehicle treated rats (adult vs. puberty: *P < 0.05*, and VEH group vs. C group on the adult stage: *P < 0.05*) while a transient but intensively increased progesterone production on the postpuberty was evidenced in the capsaicin treated rats (postpuberty vs. puberty and adult stage: *P < 0.05*) and that progesterone concentrations considerably decreased in adults from the CAP group, becoming significantly lower than values observed in the controls (*P < 0.05*). Whatever the considered developmental period, the oestrogen concentrations were significantly lowered in the VEH and CAP groups compared to the C group (*P < 0.05*) and were minimal in rats treated with the capsaicin (VEH group vs. CAP group: *P < 0.05*). Thereafter, the hormone concentrations in the 2 treated groups reached maximal values on the postpuberty period (puberty vs. postpuberty: *P < 0.05*) and remained elevated when rats were adults.

Discussion

The current study has confirmed the VR1 expression in rat ovaries whatever the sexual development period as previously described by TÜTÜNCÜ and ÖZFİLİZ [45]. Moreover, the VR1 expression in ovaries from rats treated with capsaicin at low dose was reported here for the first time.

Capsaicin is known to affect many systems in the organism, including nervous, cardiovascular, respiratory, immune and gastrointestinal systems [3, 15, 28]. Additionally, chilli and CAP have been reported to increase energy and lipid metabolisms [19, 49] and CAP has been specifically reported to reduce adiposity. This particular effect may be partly related to the CAP-induced catecholamine secretion from the adrenal medulla and the sympathetic activation of the central nervous system leading to amplification of the energy and lipid metabolisms [19, 25]. YOSHIOKA *et al*. [52-55] have shown that the CAP addition to meals increased the diet-induced thermogenesis and decreased subsequent energy intake in humans. The increase in the facultative phase of diet-induced thermogenesis was probably due to β-adrenergic stimulation [52, 53]. They have also shown a decreased appetite, a decreased cumulative food intake [54] and an increased fat oxidation and energy consumption following CAP supplementation in humans [53, 55]. In agreement with that, the mean body weight gains of the capsaicin treated rats were significantly decreased compared to the not treated controls, particularly in the postpubertal period and in adults, in the present study.

The VR1 expression was previously reported in a human bronchial epithelial cell line [46], skin epidermal keratinocytes [14], urinary bladder epithelial cells [2, 21], and rat gastric mucosal cells [31]. We have also previously demonstrated the constitutive VR1 expression in rat ovaries by immunohistochemistry [45]. Consequently, the vanilloid receptor type 1 appears to be a ubiquitous structure. On the other hand, the VR1 immunoreactivity, mainly intense in the granulosa cells and in the interstitial and corpus luteal cells at a lesser extend, was observed in the present study in the not treated control group as well as in groups treated with capsaicin or with the vehicle (10% ethanol, 10% Tween 80 and 80% distilled water) alone. The VR1 expression tended to be even strengthened in pubertal and in adults rats after capsaicin treatment at low doses. These results show that a long term capsaicin treatment at low dose does not prevent the VR1 production in rat ovaries and these findings are compatible with those of ZIK *et al*. [57]

which have demonstrated that regularly capsaicin applications at low dose did not inactivate VR1 in rat duodenum.

The effects of capsaicin on the pituitary gonad system were evoked in previous studies [8, 10, 29, 36, 44] and may be mediated by the capsaicin-induced release of neuropeptides from sensory nerves, and particularly of SP. WALCZESKA *et al.* [48] have reported the stimulatory effect of SP in the hypothalamic-pituitary system on the gonadotropin secretion and DEES *et al.* [8] have demonstrated the SP accumulation in the nerve end near the theca externa and around the blood vessels and suggested that the neuropeptide may be involved in the ovarian function by locally controlling the blood flow. In the present study, the LH concentrations were increased in the capsaicin treated rats although not significantly compared to the not treated controls except in the adult state and the FSH concentrations have remained strongly elevated until the adult period ($P < 0.05$ except at the postpuberty). As the pituitary hormones exert *in vitro* some anti-apoptotic effects on the granulosa cells and have been reported to stimulate follicle development [51], it was suggested that capsaicin at low dose for a long time was able to stimulate follicular development, leading to a positive effect on fertility. Furthermore, the increased ovary weight according to the treatment duration and the high density of developing follicles (primordial, primary and secondary follicles) observed in the capsaicin treated rats corroborated this hypothesis. Recently, ZIK *et al.* [56] have demonstrated that the capsaicin administration at low dose in prepubertal rats partially inhibited the atresia-coupled apoptosis and promoted cell proliferation. In addition, the oestrogen and progesterone concentrations were significantly depressed in the experimental group compared to the not treated control except for the progesterone concentrations at the postpuberty, suggesting a decline in the number of mature follicles and corpus luteum. However, in the postpubertal period, the peak in the progesterone coupled to the abrupt decline of FSH whereas the LH concentrations remained high suggest that ovulation may occur earlier and more rapidly than in the not treated controls.

However, in the present study, the vehicle (10% ethanol, 10% Tween 80, and 80% distilled water) injection has also affected the body weight gain, particularly at the pubertal period, and altered the ovarian structure and function by increasing the ovary weight at the adult stage and by interfering with the hormonal control and production (increases in the LH, FSH and progesterone concentrations associated to a decrease in the oestrogen concentrations). These results suggest that the vehicle used in the present study and classically used for capsaicin injections, would have increased the corpus luteum formation. These effects would be related to the presence of ethanol or of tween 80. On the other hand, the own effects of the vehicle probably interfere with the capsaicin action on the ovary structures, complicating in this way the analysis of the drug molecular actions.

As a conclusion, the present study demonstrates a constitutive expression of VR1 in rat ovaries (in granulosa, interstitial, corpus luteal cells and in the theca follicle cells at a lesser extend) independently of the sexual developmental periods and VR1/capsaicin interactions was investigated. A chronic low dose capsaicin treatment appears to promote the proliferating phases of the ovarian follicle development. However, further

studies are required for characterizing the adverse solvent effect and the specific drug actions directly on the ovarian function and indirectly on the pituitary gland.

Acknowledgement

References

1. - ADAMS E.J., GREN J.A., CLARK A.H., YOUNGSON J.H.: Comparison of different scoring systems for immunohistochemical staining. *J. Clin. Pathol.*, 1999, **52**, 75-77.

2. - BIRDER L.A., KANAI A.J., DE GROAT W.C., KISS S., NEALEN M.L., BURKE N.E., DINELEY K.E.: Vanilloid Receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 13396-13401.

3. - BIGGS D.F., LADENIUS R.C.: Capsaicin selectively reduced airway responses to histamine, substance P and vagal stimulation. *Eur. J. Pharmacol.*, 1990, **175**, 29-33.

4. - BURDEN H.W.: Ovarian innervation. *In*: The Vertebrate Ovary: Comparative Biology, JONES R.E. (ed.), Plenum Pres, New York, 1978, pp: 615-638.

5. - CALKA J., Mc DONALD J.K., OJEDA S.R.: The innervation of the immature rat ovary by calcitonin gene-related peptide. *Biol. Reprod.*, 1988, **39**, 1215- 1223.

6. - CATERINA M.J., SCHUMACHER M.A., TOMINAGA M., ROSEN T.A., LEVINE J.D., JULIUS D.: The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, 1997, **389**, 816-824.

7. - COTTON H., TRAURIG H.H., PAPKA R. : Substance P and vasoactive intestinal polypeptide-immunoreactive nerves in the female reproductive system. *Anat. Rec.*, 1983, **205**, 38A-39A.

8. - DEES W.L., KOZLOWSKI G.P., DEY R., OJEDA S.R.: Evidence for the existence of substance P in the prepubertal rat ovary. II. Immunocytochemical localization. *Biol. Reprod.*, 1985, **33**, 471-476.

9. - DOMINGUEZ R., RIBONI L.: Failure of ovulation in autographed ovary of the hemispayed rat. *Neuroendocrinology*, 1971, **7**, 164-170.

10. - ERDOST H., ÇAVU O LU I., YAKI IK M., ÖZFILIZ N., ZIK B.: Gonadotrophs in the chicken fed with a diet containing red hot pepper. *Ind. Vet. J.*, 2006, **83**, 419-423.

11. - FRITZ S., WESSLER I.R., BREITLING W., ROSSMANITH S., OJEDA R., DISSEN G.A., ÁMSTERDAM A., MAYERHOFER A.: Expresión of muscarinic receptor types in the primate ovary and evidence for nonneuronal acetylcholine synthesis. *J. Clin. Endocrinol. Metab.*, 2001, **86**, 349-354.

12. - HOLZER P.: Capsaicin: cellular targets, mechanism of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, 1991, **43**, 143-201.

13. - HOLZER P.: Neural emergency system in the stomach. *Gastroenterology*, 1998, **114**, 823-839.

14. - HUDSON L.J., BEVAN S., WOTHERSPOON G., GENTRY C., FOX A., WINTER J.: VR1 protein expression increases in undamaged DRG neurons after partial nerve injury. *Eur. J. Neurosci.*, 2001, **13**, 2105-2114.

15. - JENSEN-JAORLIM E., GAJDZIK L., HABERL I.: Hot spices influence permeability of human intestinal epithelial monolayers. *J. Nutr.*, 1998, **128**, 577-581.

16. - JESSELL T.M., IVERSEN L.L., CUELLO A.C.: Capsaicin-induced depletion of substance P from primary sensory neurones. *Brain Res.*, 1978, **152**, 183-188.

17. - KANG J.Y., ALEXANDER B., MATH M.V., WILLIAMSON R.C.: The effect of chilli and its pungent ingredient capsaicin on gastrointestinal transit in the rat. *J. Gastroenterol. Hepatol.*, 1993, **8**, 513-516.

18. - KANNISTO P., EKBLAD E., HELM G., OWMAN C., SJÖBERG N.O., STJERNQUIST M., SUNDLER F., WALLES B.: Existence and coexistence of peptides in nerves of the mammalian ovary and oviduct demonstrated by immunocytochemistry. *Histochemistry*, 1986, **86**, 25-34.

19. - KAWADA T., SAKABE S., WATANABE T., YAMAMOTO M., IWAI K.: Some pungent principles of spices cause the adrenal medulla to secrete catecholamine in anesthesized rats. *Proc. Soc. Exp. Biol. Med.*, 1988, **188**, 229-233.

20. - KLEIN C.M., BURDEN H.W.: Substance P and vasoactive intestinal polypeptip (VIP) immunoreactive nerve fibers in relation to ovarian postganglionic perikarya in para and prevertebral ganglia: evidence from combined retrograde tracing and immunocytochemistry. *Cell Tissue Res.*, 1988, **252**, 403-410.

21. - LAZZERIA M., VANNUCCHIC M.G., ZARDOC C., SPINELLIB M., BENEFORTIA P., TURINIA D., FAUSSONE-PELLEGRINIC M.S.: Immunohistochemical evidence of vanilloid receptor 1 in normal human urinary bladder. *Eur. Urol.*, 2004, **46**, 792-798.

22. - MAGGI C.A., MANZINI S., GIULIANI S., SANTICIOLI P., MELI A.: Extrinsic origin of the capsaicin-sensitive innervation of rat duodenum: possible involvement of calcitonin-gene related peptide (CGRP) in the capsaicin-induced activation of intramural non-adrenergic non-cholinergic neurons. *Naunyn-Schmiedeberg Arch. Pharmacol.*, 1986, **334**, 172-180.

23. - MARINELLI S., VAUGHAN C.W., Mc DONALD J.C., CONNOR M.: Capsaicin activation of glutamatergic synaptic transmission in the rat locus coeruleus *in vitro*. *J. Physiol.*, 2002, **5432**, 531-540.

24. - MARINELLI S., PASCUCCI T., BERNARDI G., PUGLISI-ALLEGRA S., MERCURI N.B.: Activation of TRPV1 in the VTA excites dopaminergic neurons and increases chemical-and noxiousinduced dopamine release in the *nucleus accumbens*. *Neuropsychopharmacology*, 2005, **30**, 864-870.

25. - MATSUO T., YOSHIOKA M., SUZUKI M.: Capsaicin in diet does not affect glycogen contents in the liver and skeletal muscle of rats before and after exercise. *J. Nutr. Sci. Vitaminol.*, 1996, **42**, 249-256.

26. - MAYERHOFER G.A., DISSEN M., COSTA E., OJEDA S.R.: A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. *Endocrinology*, 1997, **138**, 3320-3329.

27. - MEZEY E., TOTH Z.E., CORTRIGHT D.N., ARZUBI M.K., KRAUSE J.E., ELDE R., GUO A.: Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 3655-3660.

28. - MONSEREENUSORN Y.: Subchronic toxicity studies of capsaicin and capsicum in rats. *Res. Commun. Chem. Pathol. Pharmacol.*, 1983, **41**, 95-110.

29. - MORAN C., MORALES L., RAZO S.R., APOLONIO J., QUIROZ U., CHAVIRA R., DOMINGUEZ R.: Effect of sensorial denervation induced by *capsaicin* ınjection at birth or on day three of life, on puberty, ınduced ovulation and pregnancy. *Life Sci.*, 2003, **73**, 2113-2125.

30. - NAGY J.I.: Capsaicin: a chemical probe for sensory neuron mechanisms. *In*. Handbook of psychopharmacology, Iversen L.L., Iversen S.D., Snyder S.H. (eds), Plenum Press, New York, 1982, pp.: 185-235.

31. - NOZAWAY., NISHIHARA K., YAMAMOTO A., NAKANO M., AJIOKA H., MATSUUR N.: Distribution and characterization of vanilloid receptors in the rat stomach. *Neurosci. Lett.*, 2001, **309**, 33-36.

32. - OJEDA S.R., COSTA M.E., KATZ K.H., HERSH L.B.: Evidence for the existence of substance P in the pre-pubertal rat ovary. I. Biochemical and physiological studies. *Biol. Reprod.*, 1985, **33**, 286-295.

33. - OLIVEROS L., FORNERIS M., AGUADO L.: Secretion from neuropeptide-treated splenocytes modifies ovarian steroidogenesis. *Medicina*, 2001, **61**, 35-39.

34. - OLSON L., JONES B.C.H., JABLONKA-SHARIFF A.: Nitric oxide decreases estradiol synthesis of rats luteinized ovarian cells: possible role for nitric oxide in functional luteal regression. *Endocrinology*, 1996, **137**, 3531-3536.

35. - PAPKA R.E., COTTON J.P., TRAURIG H.H.: Comparative distribution of neuropeptide tyrosine-, vasoactive intestinal polypeptide-, substance P immunoreactive, acetylcholinesterase-positive and noradrenergic nerves in the reproductive tract of the female rat. *Cell Tissue Res.*, 1985, **242**, 475-490.

36. - PINTADO C.O., PINTO F.M., PENNEFATHER J.N., HIDALGO A., BAAMONDE A., SANCHEZ T., CANDENAS M.L.: A role for tachykinins in female mouse and rat reproductive function. *Biol. Reprod.*, 2003, **69**, 940-946.

37. - SARIA A., MA R.C., DUN N.J., THEODORSSON-NORHEIM E., LUNDBERG J.M.: Neurokinin A in capsaicin-sensitive neuronsof the guinea-pig inferior mesenteric ganglia: an additional putative mediator for the non-cholinergic excitatory postsynaptic potential. *Neuroscience*, 1987, **21**, 951-958.

38. - SMITH M.S., FREEMAN M.E., NEILL J.D.: The control of progesterone secretion during the cycle and early pseudopregnancy. *Endocrinology*, 1975, **96**, 219-226.

39. - SOSA Z.Y., CASAIS M., RASTRILLA A.M., AGUADO L.I.: Adrenergic ınfluences on coeliac ganglion affect the release of progesterone from cycling ovaries. Characterization of an *in vitro* system. *J. Endocrinol.*, 2000, **166**, 307-318.

40. - STÄNDER S., MOORMANN C., SCHUMACHER M., BUDDENKOTTE J., ARTUC M., SHPACOVITCH V., BRZOSKA T.: Expression of vanilloid receptor subtype 1 in cutaneous sensory nerve fibers, mast cells, and epithelial cells of appendage structures. *Exp. Dermatol.*, 2004, **13**, 129-139.

41. - SURH Y.J., LEE S.S.: Capsaicin in hot chili pepper: carcinogen, cocarcinogen or anticarcinogen? *Food Chem. Toxicol.*, 1996, **34**, 313-316.

42. - SZALLASI A., BLUMBERG P.M.: Vanilliod (capsaicin) receptors and mechanisms. *Pharmacol. Rev.*, 1999, **51**, 159-211.

43. - SZOLCSANYI J., BARTHO L.: Capsaicin-sensitive afferents and their role in gastroprotection: an update. *J. Physiol.*, 2001, **95**, 181-188.

44. - TRAURIG H.H., SARIA A., LEMBECK F.: The effects of neonatal capsaicin treatment on growth and subsequent reproductive function in the rat. Naunyn Schmiedebergs Arch. *Pharmacol.*, 1984, **327**, 254-259.

45. - TÜTÜNCÜ S., ÖZFİLİZ N.: Distribution of the vanilloid (capsaicin) receptor type 1 in the rat ovaries on different sexual development period. *Rev. Med. Vet.*, 2010, **161**, 272-276.

46. - VERONESI B., OORTGIESEN M., CARTER J.D., DEVILN R.B.: Particulate matter initiates inflammatory cytosine release by activation of capsaicin and acid receptors in a human bronchial epithelial cell line. *Toxicol. Appl. Pharmacol.*, 1999, **154**, 106-115.

47. - VLACHOVA V., TEISINGER J., SUSANKOVA K., ETTRICH R., VYKLICKY L.: Functional role of C-terminal cytoplasmic tail of rat vanilloid receptor 1. *J. Neurosci.*, 2003, **23**, 1340-503.

48. - WALCZEWSKA A., SZKUDLAREK U., JAKUBOWSKA-NAZIEMBLO B., DZIEDZIC B., WLADYSLAW Z.: Increase of gonadotropin-releasing hormone concentration in pituitary portal blood after substance p administration in male rats. *Neuroendocrinology*, 1998, **67**, 219-227.

49. - WARDLE K.A., RANSON J., SANGER G.J.: Pharmacological characterization of the vanilloid receptor in the rat dorsal horn. *Br. J. Pharmacol.*, 1997, **121**, 1012-1016.

50. - WATANABE T., KAWADA T., IWAI K.: Enhancement by capsaicin of energy metabolism in rats through secretion of catecholamine from adrenal medulla. *Agric. Biol. Chem.*, 1987, **51**, 75-79.

51. - YACOBI K., WOJTOWICZ A., TSAFRIRI A., GROSS A.: Gonadotropin enhance caspase-3 and -7activity and apoptosis in the thecaınsterstitial cells of rat preovulatory follicles in culture. *Endocrinology*, 2004, **145**, 1943-1951.

52. - YOSHIOKA M., LIM K., KIKUZATO S.: Effects of red-pepper diet on the energy metabolism in men. *J. Nutr. Sci. Vitaminol.*, 1995, **41**, 647-656.

53. - YOSHIOKA M., ST-PIERRE S., SUZUKI M., TREMBLAY A.: Effects of red pepper added to high-fat and high-carbohydrate meals on energy metabolism and substrate utilization in Japanese women. *Br. J. Nutr.*, 1998, **80**, 503-510.

54. - YOSHIOKA M., ST-PıERRE S., DRAPEAU V.: Effects of red pepper on appetite and energy intake. *Br. J. Nutr.*, 1999, **82**, 115-123.

55. - YOSHIOKA M., DOUCET E., DRAPEAU V., DIONNE I., TREMBLAY A.: Combined effects of red pepper and caffeine consumption on 24 h energy balance in subjects given free access to foods. *Br. J. Nutr.*, 2001, **85**, 203-211.

56. - ZIK B., ÖZGÜDEN C., TÜTÜNCÜ Ş., İLHAN T.: Expression of vanilloid receptor-1 in the duodenum of the capsaicin treated rat. *Bull. Vet. Inst. Pul.*, 2007, **51**, 149-153.

57. - Zık B., Özgüden Akkoç C.G., Tütüncü S., İlhan T., Yılmaztepe A.O., Özenci C.C.: Effects of low dose capsaicin (CAP) on ovarian follicle development in prepubertal rat. *Rev. Med. Vet.*, 2010, **161**, 288-294.