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Local VEGF inhibition prevents ovarian alterations associated with ovarian hyperstimulation syndrome

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ABSTRACT

The relationship between human chorionic gonadotropin and ovarian hyperstimulation syndrome (OHSS) is partially mediated by vascular endothelial growth factor A (VEGF). The aim of this study was to investigate the effects of VEGF inhibition on the development of corpora lutea (CL) and cystic structures, steroidogenesis, apoptosis, cell proliferation, endothelial cell area, VEGF receptors (KDR and Flt-1), claudin-5 and occludin levels in ovaries from an OHSS rat model. The VEGF inhibitor used (VEGF receptor-1 (FLT-1)/Fc chimera, TRAP) decreased the concentrations of progesterone and estradiol as well as the percentage of CL and cystic structures in OHSS rats, and increased apoptosis in CL. Endothelial cell area in CL and KDR expression and its phosphorylation were increased, whereas claudin-5 and occludin levels were decreased in the OHSS compared to the control TRAP reversed these parameters. Our findings indicate that VEGF inhibition prevents the early onset of OHSS and decreases its severity in rats.

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1. Introduction

Ovarian hyperstimulation syndrome (OHSS) remains the most serious complication of gonadotropin treatment. OHSS occurs in 5%–10% of patients undergoing ovulation induction therapy, and its severe form takes place in 0.5%–5.0% of these patients [1,2]. Despite advances in prediction and management of OHSS, complete prevention has not been possible yet [3,4].

The main clinical components of this syndrome are marked enlargement of the ovaries, with luteal and hemorrhagic cysts, and an excessive fluid shift [5]. This shift is caused by increased vascular permeability in response to stimulation with human chorionic gonadotropin (hCG) [6].

Prostaglandins, inhibin, the renin–angiotensin–aldosterone system and inflammatory mediators have all been implicated in the etiology of OHSS [7]. However, vascular endothelial growth factor A (VEGF) has been identified as the main mediator [8]. The receptors for VEGF are localized in endothelial cells and belong to the tyrosine kinase receptor family. Two membrane receptors for VEGF, VEGF receptor-1 (Flt-1) and VEGF receptor-2 (Flk-1/KDR), have been identified in endothelial cells. KDR is mainly involved in regulating vascular permeability and angiogenesis [9,10]. VEGF receptors are also present in ovarian follicles and corpora lutea from rats, monkeys and humans [11–15]. The mRNA expression of VEGF and KDR increases significantly in human granulosa-lutein cells in response to hCG, and its peak levels coincide with maximum vascular permeability [6]. In women who develop OHSS, VEGF is produced by granulosa-lutein cells and endothelial cells in response to hCG. VEGF is then released into the follicular fluid, inducing increased capillary permeability in an autocrine and paracrine manner [16–18].

Several authors have shown that the ovary requires tightly controlled dynamic changes in the localization and expression of adhesion molecules [19,20]. Major components of intercellular junctions are adherens and tight junction proteins [21,22]. An increase in endothelial permeability is generally accompanied by reorganization of junctional proteins, prompting a transient opening of the endothelial junctions and a subsequent increase in paracellular permeability. Tight junctions (TJ) are formed by homotypic or heterotypic binding of the amino terminal domains of transmembrane adhesion molecules, such as claudin, occludin and junctional adhesion molecules from the adjacent endothelial cells. Claudin-5, which is specific of endothelium, is one of the main claudins [23,24]. Occludin is another transmembrane protein structurally similar to claudins, which becomes incorporated into claudin-based junctional strands [25,26]. In the ovary, Rodewald et al. demonstrated that TJ proteins are differentially expressed during follicular development, which may indicate that these

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proteins play a role in the regulation of follicular growth, antrum transition and ovarian angiogenesis [20]. In addition, VEGF signaling is thought to mediate the redistribution of TJ proteins and the loss of the endothelial cell barrier architecture [27]. In particular, in OHSS, Rodewald et al. showed that hCG can increase endothelial permeability by up-regulating VEGF in human luteinized granulosa cells, which causes a decrease in endothelial claudin-5 expression [23].

Several studies have shown that VEGF concentration in follicular fluid is 100-fold greater than in serum or peritoneal fluid of patients considered at risk for OHSS [28–30]. This increased concentration of VEGF in follicular fluid suggests that the ovary is a significant source of VEGF.

In our laboratory, we have previously demonstrated that inhibition of VEGF by intrabursal administration of the VEGF inhibitor TRAP causes an imbalance in the ratio between antiapoptotic and proapoptotic proteins, which leads a larger number of follicles to atresia in the rat ovary [31,32]. So far, there are no studies on the effect of *in vivo* VEGF inhibition on the ovarian morphology and vascular development in a rat model of OHSS.

In the present study, we hypothesized that the alteration in VEGF levels observed in OHSS may be in part responsible for the ovarian dysfunction (luteal development, luteal angiogenesis, steroidogenesis, apoptosis, TJ proteins), which is the main feature of this syndrome. Therefore, the main purpose of this study was to investigate the effect of VEGF inhibition on luteal development, formation of cystic structures, steroidogenesis, apoptosis and proliferation in ovaries from a rat OHSS model. In addition, we evaluated the effect of VEGF inhibition on endothelial cell area, and on the protein expression of VEGF receptors (KDR and Flt-1), claudin-5 and occludin in ovaries from this model.

2. Materials and methods

2.1. Hormones and drugs

Recombinant rat soluble VEGF receptor 1/Fc Chimera (TRAP) (R&D Systems Inc., Minneapolis, MN, USA) was dissolved in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) from Sigma–Aldrich (St. Louis, MO, USA). Pregnant mare serum gonadotropin (PMSG) (Novormon) was provided by Syntex S.A. (Buenos Aires, Argentina) and hCG (Endocorion) by Elea (Buenos Aires, Argentina). Polyclonal primary antibody for B actin (sc-1616), p-Akt (sc-7985-R), PCNA (sc-7907) and Flt-1 (sc-9029) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); cleaved Caspase-3 (CP229) was from Biocare Medical (Concord, CA, USA); claudin-5 (35–2500) and occludin (71–1500) were from Invitrogen Corp. (Carlsbad, CA, USA); and Akt (9272), phospho-KDR (19A10) and KDR (55B11) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase and lectin from *Bandeiraea simplicifolia* biotin conjugate were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade and were obtained from standard commercial sources.

2.2. Animal model and experimental design

Rats were housed and cared at the Instituto de Biología y Medicina Experimental (IByME), Buenos Aires, Argentina. Immature female Sprague-Dawley rats (21–23 days old) from our colony ($n = 6$/group for each treatment) were allowed food and water *ad libitum* and kept at room temperature (21–23 °C) on a 12L:12D cycle. All protocols and experiments were approved by the ethics committee of the IByME and conducted according to the guide for the care and use of laboratory animals of the National Institute of Health (USA). We used an animal model that develops OHSS in immature Sprague-Dawley rats (21–23 days old, 60–80 g) as described by Kitajima et al. [33,34]. The rats were randomly divided into six groups: the control groups (control 48 h and control 72 h), injected with PMSG (10 UI) on the 23rd day of life at 9 h, and with hCG 48 h later (10 UI); the OHSS groups (OHSS 48 h and OHSS 72 h), injected with excessive doses of PMSG (50 UI/day) for 4 consecutive days (from the 21st to the 24th day of life, at 9 h), followed by hCG (25 UI, 25th day of life at 9 h); and the OHSS + TRAP groups (OHSS + TRAP 48 h and OHSS + TRAP 72 h), injected with the same doses of gonadotropins and hCG as the OHSS groups and then treated with TRAP (25 UI, 25th day of life at 9 h). To inhibit VEGF, TRAP was administered under the bursa of the ovaries. The animals were anesthetized with ketamine HCl (70 mg/kg; Holliday-Scott S.A., Buenos Aires, Argentina) and xylazine (5 mg/kg; König Laboratories, Buenos Aires, Argentina). The ovaries were exteriorized through an incision made in the dorsal lumbar region. The OHSS + TRAP groups then received 1 μg in 5 μl PBS/0.1% BSA of TRAP under the bursa of both ovaries, whereas the other groups of animals received the vehicle solution. Rats were then killed by decapitation 48 h and 72 h after the hCG injection for ovary and blood collection. The ovaries were removed and cleaned of adhering tissue in culture medium, weighed, and used for subsequent assays. The serum was used for hormone assays. One of the ovaries from each of the six rats from each of the six groups ($n = 6$) was used for Western immunoblot assay and the other for immunohistochemical assay.

2.3. Steroid hormone assay

Serum steroid concentrations were measured by radioimmunoassay (RIA) ($n = 6$ rats/group) [35,36]. Progesterone (P_4) and estradiol (E_2) were measured by using specific antibodies supplied by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA). Under these conditions, the intraassay and interassay variations were 8.0% and 14.2% for P_4 and 7.2% and 12.5% for E_2. The values are expressed per ml of serum.

2.4. Ovarian morphology

Ovaries were extracted from the different experimental groups and immediately fixed in paraformaldehyde 4% for 12 h. Histological sections were made for staining with hematoxylin–eosin (H&E). Sections (5 μm) were mounted at 50 μm intervals onto microscope slides to prevent counting the same structure twice, according to the method described by Woodruff et al. [37]. Atretic follicles (Atret. F), corpora lutea (CL) and cystic structures were counted per ovary section. The number of these different ovarian structures was determined in six ovarian sections from each ovary ($n = 6$ ovaries/group) and expressed as percentage of Atret. F, CL or cysts/ovary. The total number of ovarian structures was defined as 100%. An Atret. F was defined as the follicle that presented more than 10 pycnotic nuclei per follicle [38,39]. Cysts were defined as structures with presence of oocytes surrounded by luteal cells, remaining granulosa cells and red blood cells.

2.5. Histochemistry and immunohistochemistry in luteal tissues

Tissue sections were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS and nonspecific binding was blocked with 2% BSA overnight at 4 °C. Sections were incubated with rabbit polyclonal cleaved caspase-3 (1/100) or biotinylated lectin BS-1 (from *Bandeiraea simplicifolia*, 20 μg/ml) overnight at 4 °C. Lectin BS-1 has been proved to be a constitutive endothelial cell marker staining endothelial cells at the different

developmental stages of CL with similar intensity [40–42]. After washing, the slides were incubated with biotinylated anti-rabbit IgG (except in the case of lectin BS-1) and afterwards with avidin-biotinylated horseradish peroxidase Complex (Vectastain ABC system from Vector Laboratories, Burlingame, CA, USA) for 30 min. Protein expression was visualized with diaminobenzidine (DAB) staining. The reaction was stopped with distilled water, stained with methylene green or hematoxylin and dehydrated before mounting (Canada Balsam Synthetic, Biopack, Argentina). Negative controls were obtained in the absence of primary antibody.

To assess cell death in cleaved caspase-3-stained sections, we analyzed three fields randomly selected from each ovarian section (six sections/ovary, six ovaries/group). Immunopositive cells were expressed as "number of cleaved caspase-3-positive cells/tissue area". The images were digitized using a camera (Nikon, Melville, NY, USA) mounted on a conventional light microscope (Nikon), using a magnification of 400X. These microphotographs were analyzed using the software Image J (Image Processing and Analysis in Java, National Institute of Health, USA), using the Cell Counter Tool. For sections stained for lectin BS-1, six sections were analyzed per ovary (six ovaries/group) and three randomly selected fields in each section were photographed. Images were converted to TIFF format (bi-level scale) for their analysis and processed using Image Pro® Plus 3.0 (Media Cybernetics, Silver Spring, MA, USA). Vascular area (lectin BS-1-positive cells) was determined by thresholding the lectin BS-1-positive stained area and calculated by relativization to the total luteal area of the captured photograph. The microphotographs were analyzed by an observer blinded to the treatment type.

2.6. Western blot

Ovaries were removed, placed on ice and resuspended in five volumes of lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-p-tosyl-lysine chloromethyl ketone and 0.025 m-L-1-tosylamide-2-phenyl-ethylchloromethyl ketone) and homogenized with an Ultra-Turrax homogenizer (IKA Werk, Breisgau, Germany). Samples were centrifuged at 4 °C for 10 min at 10,000 × *g* and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 min, 40 mg of protein was applied to a 12% SDS-polyacrylamide gel and electrophoresis was performed at 25 mA for 1.5 h. The resolved proteins were transferred onto nitrocellulose membranes for 2 h. The blot was preincubated in blocking buffer (5% nonfat milk, 0.05% Tween-20 in 20 mM TBS pH 8.0) for 1 h at room temperature and incubated overnight with appropriate primary antibodies (PCNA: 1/200, Akt: 1/500, p-Akt: 1/1000, occludin: 1/1000, claudin-5: 1/2000; p-KDR: 1/1000 and KDR: 1/500 in TBS) in blocking buffer at 4 °C. The blot was then incubated with anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:1000) and finally detected by chemiluminescence and autoradiography using X-ray film. The density in each band was normalized to the density of the B actin band, which was used as an internal control. Equal amounts of protein were loaded for all samples, and different groups in one experiment were loaded on the same gel. The levels of protein were compared and analyzed by densitometric studies using Scion Image for Windows (Scion Corporation, Worman's Mill Ct., MD, USA). Optical density data are expressed as arbitrary units ± SEM ($n = 6$).

2.7. Data analysis

The results are expressed as the mean ± SEM and the significant differences between groups were determined using analysis of variance (ANOVA), followed by Tukey's test or the multiple comparison Newman–Keuls test. P values <0.05 were considered statistically significant. All samples were tested for normality before ANOVA. Data were statistically analyzed using Prism v5.0.

3. Results

3.1. Measurement of ovarian weight and serum steroids

The effects of TRAP-treatment on ovarian weight and steroid hormone serum concentrations are summarized in Table 1. The ovarian weight is expressed as the weight of individual ovary. The ovarian weight in OHSS rats was greater than that in control rats at both time points analyzed ($p < 0.05$). At 72 h, the administration of TRAP significantly reduced the ovarian weight as compared with that observed in the OHSS group without treatment ($p < 0.05$).

At 48 and 72 h after the hCG injection, the concentrations of serum E_2 significantly increased compared to the control groups ($p < 0.05$). After 48 h, TRAP treatment decreased the concentrations of serum E_2 and P_4 compared to the OHSS group without treatment ($p < 0.05$), whereas after 72 h, TRAP-treatment decreased E_2 concentrations compared to the OHSS group without treatment ($p < 0.05$).

3.2. Ovarian morphology

We have previously observed that TRAP treatment results in an increased percentage of atretic follicles compared to the OHSS group without treatment (48 h: OHSS = 0.28 ± 0.08, OHSS + TRAP = 9.76 ± 1.5; 72 h: OHSS 72 h = 2.88 ± 1.09, OHSS + TRAP = 7.51 ± 2.71, $p < 0.01$) (data not shown). Ovarian morphology in OHSS rat model is presented in Fig. 1A. The percentage of CL in OHSS rats was higher than that in control rats (48 h: $p < 0.01$; 72 h: $p < 0.05$). TRAP treatment decreased the percentage of CL in the ovaries from OHSS rats compared to the untreated groups at 48 h and 72 h (48 h: $p < 0.05$; 72 h: $p < 0.05$) (Fig 1B). At 48 and 72 h, TRAP-treatment reduced the percentage of cystic structures in OHSS rats compared to untreated ones (48 h: $p < 0.05$; 72 h: $p < 0.05$) (Fig 1C).

Table 1
Effects of *in vivo* VEGF inhibition by TRAP on ovarian weight and serum hormone concentrations in a rat ovarian hyperstimulation syndrome (OHSS) model.

	Control 48 h ($n = 6$)	OHSS 48 h ($n = 6$)	OHSS + TRAP 48 h ($n = 6$)	P value	Control 72 h ($n = 6$)	OHSS 72 h ($n = 6$)	OHSS + TRAP 72 h ($n = 6$)	P value
Ovarian weight (g)	0.132 ± 0.013 [a]	0.217 ± 0.009 [b]	0.208 ± 0.009 [b]	<0.05	0.125 ± 0.006 [a]	0.221 ± 0.009 [b]	0.185 ± 0.003 [a]	<0.05
Serum estradiol (ng/ml)	0.356 ± 0.058 [a]	0.790 ± 0.110 [b]	0.415 ± 0.120 [a]	<0.05	0.149 ± 0.005 [a]	0.280 ± 0.030 [b]	0.130 ± 0.035 [a]	<0.05
Serum progesterone (ng/ml)	133.00 ± 45.46 [a]	389.20 ± 48.36 [b]	243.30 ± 28.52 [a]	<0.05	100.90 ± 12.04 [a]	421.30 ± 59.70 [b]	399.90 ± 78.01 [b]	<0.05

Data are expressed as mean ± SEM; $n = 6$ rats/group. Letters indicate a significant statistical difference between groups of the same time, by one-way ANOVA, followed by Tukey's multiple comparison test.

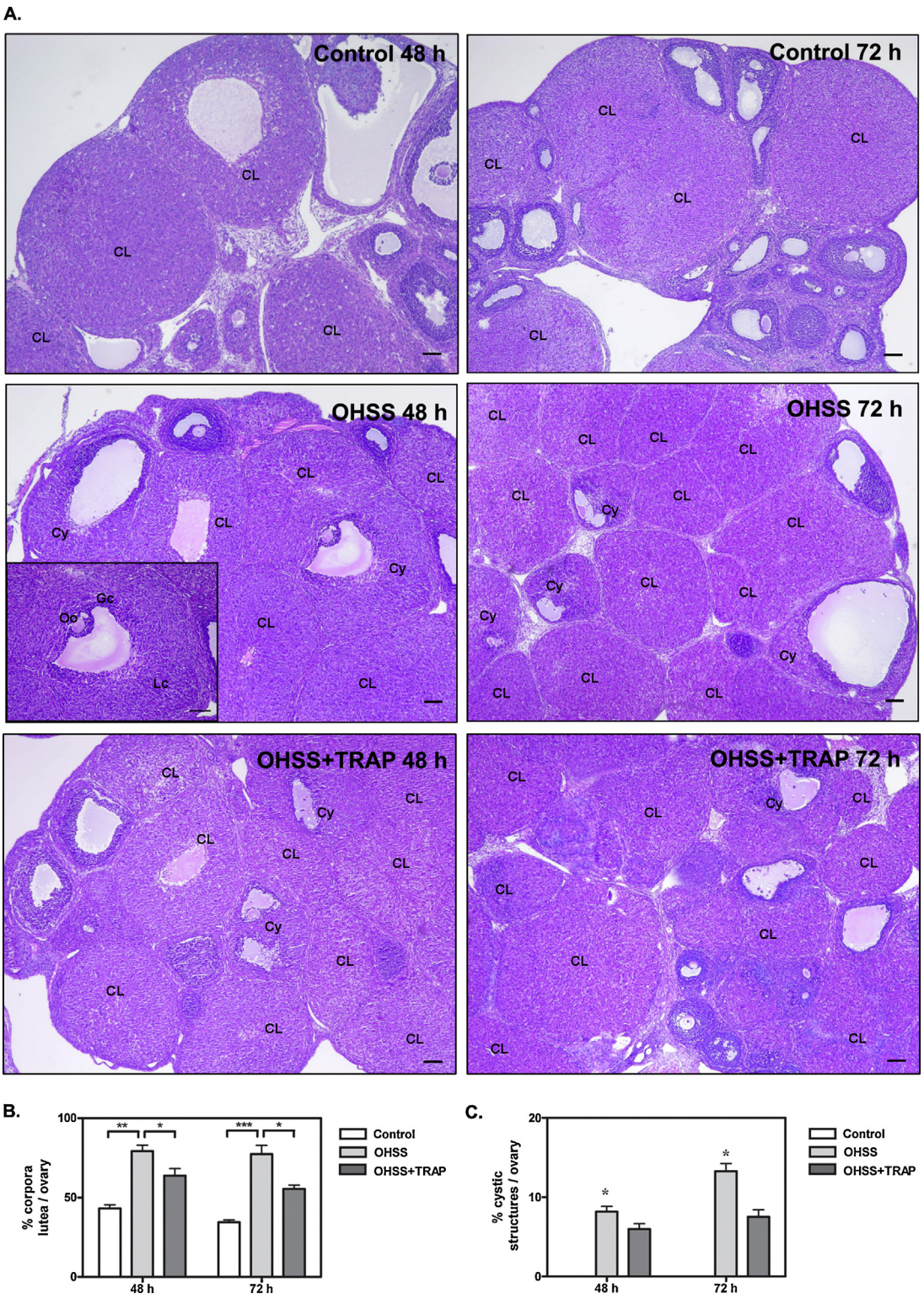

Fig. 1. Effect of TRAP treatment on ovarian morphology in a rat OHSS model. (A) Representative fields of ovarian sections stained with H&E in the six experimental groups. Original magnification 100X. Inset shows a cystic structure, original magnification 400X. Scale bars represent 50 μm. CL: corpus luteum; Cy: cyst; Oo: oocyte; Gc: granulosa cell; Lc: luteal cell. Corpora lutea (CL) and cystic structures were counted in randomly selected fields from each ovarian section (6 sections/ovary, $n = 6$ ovaries/group) and expressed as (B) percentage of CL/ovary or (C) percentage of cysts/ovary. Data are expressed as the mean ± SEM ($^{*}p < 0.05$; $^{**}p < 0.01$).

3.3. Effects of TRAP-treatment on ovarian characteristics

To analyze whether TRAP-treatment has an inhibitory effect in cell growth, we measured PCNA levels in ovaries from our OHSS model by Western blot. The results showed an increase in ovarian PCNA content in OHSS rats compared to the control group at both time points analyzed (48 h: $p < 0.05$; 72 h: $p < 0.05$). TRAP-treatment decreased ovarian PCNA expression after 48 h and 72 h of treatment compared to untreated OHSS rats ($p < 0.05$) (Fig. 2).

To evaluate luteal apoptosis in the ovaries from our OHSS model (Fig. 3), we performed immunohistochemistry (IHC) for cleaved caspase-3 (active) and quantified the number of positive nuclei in

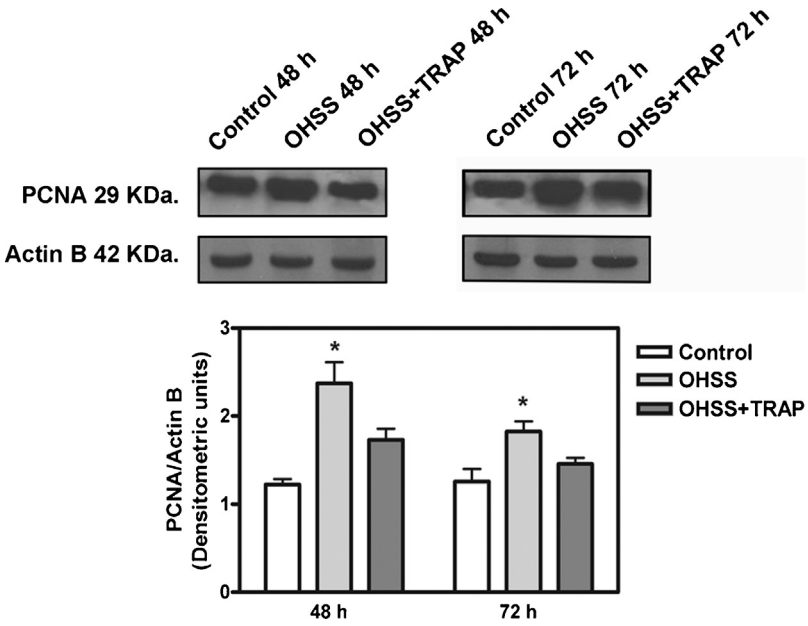

Fig. 2. Effect of TRAP treatment on ovarian cell proliferation in a rat OHSS model. Representative immunoblots of PCNA expression and densitometric quantification of PCNA. Optical density is expressed as arbitrary units±SEM normalized to B actin ($n = 6$/group, $^{*}p < 0.05$).

the total number of cells. The treatment with TRAP after 48 h and 72 h increased the percentage of apoptotic cells compared to the OHSS groups ($p < 0.01$). No changes were observed between the control and OHSS groups.

To evaluate whether TRAP-treatment causes changes in endothelial cell density, we performed a staining for lectin BS-1 in ovarian sections from our OHSS model (Fig. 4). The figure shows an increase in the binding of lectin BS-1 in CL from the OHSS groups compared to the control groups (48 h: $p < 0.01$; 72 h: $p < 0.05$). VEGF inhibition reduced the lectin binding in the CL from OHSS rats compared to OHSS rats without treatment (48 h: $p < 0.01$; 72 h: $p < 0.05$).

Also, to examine whether VEGF is involved in the phosphorylation of AKT in ovaries from our OHSS model, we measured the phosphorylated form of AKT (pAKT) at different times after TRAP-treatment (Fig. 5E and F). The protein levels of pAKT were increased at 48 h and 72 h in ovaries from the OHSS groups compared to the control groups ($p < 0.05$). Administration of TRAP decreased AKT phosphorylation compared to the untreated OHSS groups and this effect was observed 48 h and 72 h after the treatment ($p < 0.05$).

Since VEGF signaling is thought to mediate redistribution of TJ proteins and the loss of the endothelial cell barrier architecture, we decided to study the ovarian expression of the two TJ proteins claudin-5 and occludin in this OHSS model (Fig. 6A and B). In the OHSS groups, the levels of claudin-5 decreased compared to the controls (48 h: $p < 0.01$; 72 h: $p < 0.001$). However, the treatment with TRAP prevented the decrease in claudin-5 expression compared to the OHSS groups without treatment ($p < 0.05$). Inhibition of VEGF increased the levels of occludin compared to the OHSS groups without treatment (48 h: $p < 0.05$; 72 h: $p < 0.01$) (Fig. 6B).

4. Discussion

We have previously shown the effect of the local administration of a VEGF inhibitor on follicular development and apoptosis in eCG-treated prepubertal rats [11,43]. In the present study, we demonstrated for the first time that VEGF inhibition via *in vivo* intrabursal administration of TRAP affects ovarian weight, steroidogenesis, luteal development and the formation of cystic structures, increases the expression of active caspase-3 and decreases cell proliferation in the ovaries from an immature rat OHSS model. Besides, we observed that local inhibition of VEGF caused a decrease in endothelial area, KDR expression and its phosphorylation, and an increase in claudin-5 and occludin protein levels in the ovaries of the above-mentioned model.

The OHSS experimental model used in this study was useful owing to the similarity between the rat and human VEGF systems [13]. Levin et al. showed that the administration of hCG increases vascular permeability and ascitis (two of the clinical symptoms of OHSS) in the rat OHSS model [44]. For this reason, this model has been used by several authors and our group [33,34,45–47].

Several clinical studies have demonstrated the efficiency of VEGF inhibition using a soluble "decoy" receptor VEGF in other disorders. For example, Gharbiya et al. and Chang et al. have shown the effect of the VEGF inhibitor Aflibercept (VEGF Trap-Eye Regeneron-Bayer) on neovascular age-related macular degeneration [48,49].

In humans, OHSS causes the formation of multiple CL and an increase in the levels of VEGF [50]. In this study, we showed that in ovaries from OHSS rats, the inhibition of VEGF caused a decrease in the percentage of CL at 48 h and 72 h of treatment, reaching values similar to those of the control groups. These results suggest that TRAP may decrease the percentage of CL, which are able to secrete several angiogenic factors, such as VEGF, which in turn favors the altered angiogenesis and vascular permeability observed in OHSS. VEGF inhibition is likely to improve this important aspect of OHSS. In addition, we have previously observed a decrease in the percentage of atretic follicles in OHSS rats compared to control ones [47]. TRAP treatment resulted in an increase in the percentage of atretic follicles compared to OHSS rats without treatment (data not shown). This result suggests that the inhibition of VEGF causes an increase in the percentage of atretic follicles present in the ovary, and thus a decrease in the number of follicles available to ovulate. Several studies have shown that the main event that causes the regression of CL is luteal cell death by apoptosis [51]. Based on the results of ovarian morphology observed in this study and knowing that VEGF inhibition possesses a direct apoptotic effect on ovarian cells [11,31], we evaluated the active form of caspase-3, a key effector protease

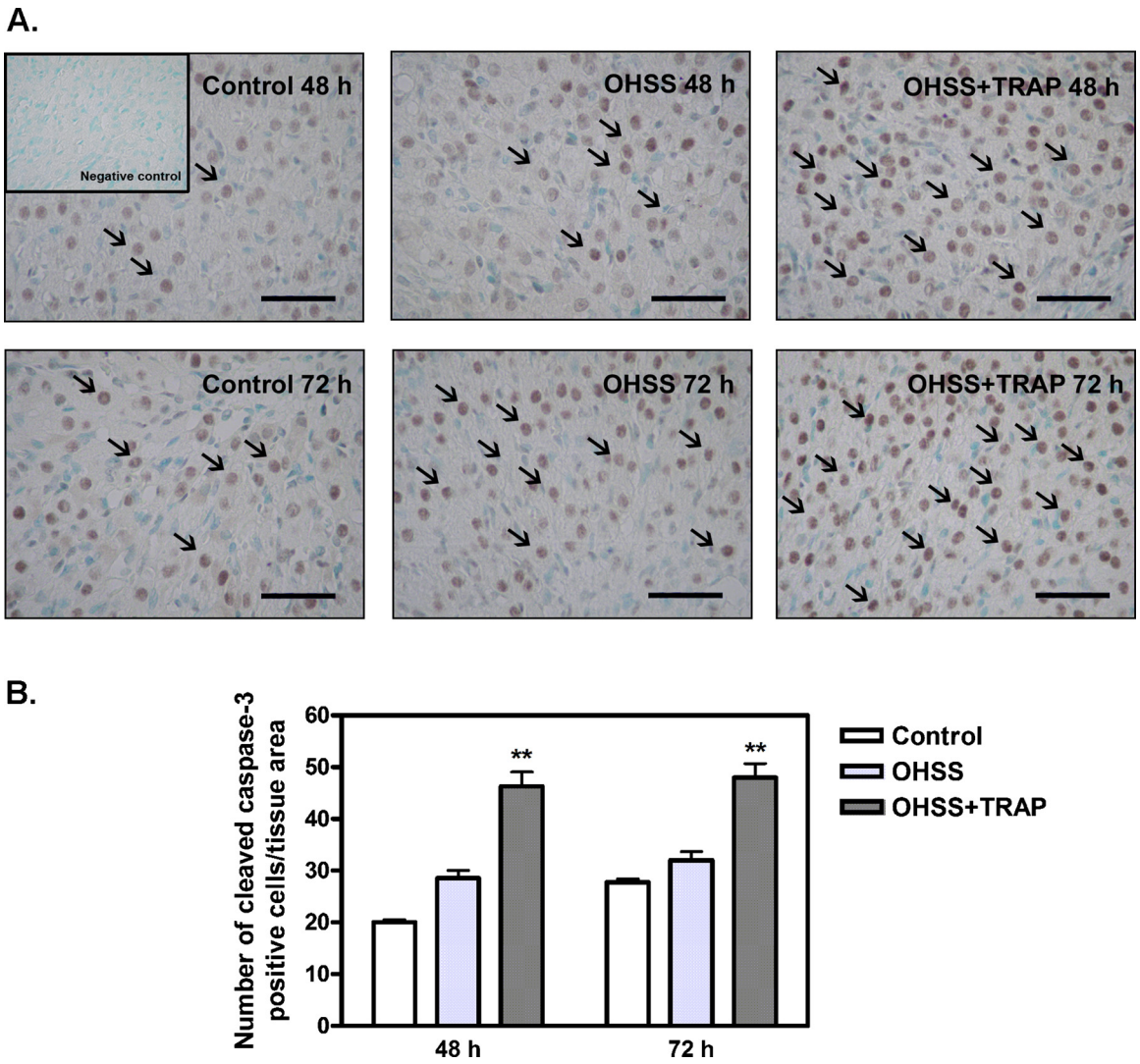

Fig. 3. Effect of TRAP treatment on cleaved caspase-3 expression in CL in a rat OHSS model. (A) Photographs show representative fields of ovarian sections immunostained for cleaved caspase-3 in the six experimental groups. The arrows show positive nuclei for cleaved caspase-3. Original magnification 400X. Scale bars represent 50 μm. The inset shows the negative control. (B) The expression of cleaved caspase-3 was determined by counting labeled cells in CL at 400X in randomly selected fields of CL. Data are expressed as mean ± SEM. Six sections per CL, ten CL per ovary and six ovaries per group were analyzed ($n = 6$ ovaries/group, $^{*}p < 0.05$).

involved in the apoptotic cascade, in OHSS ovaries. TRAP administration caused an increase in the percentage of apoptotic cells in CL compared to that observed in the untreated OHSS groups. All these findings suggest that the decrease in the percentage of CL would be caused by the regression of CL and/or by an alteration in the ovulation rate. These results are consistent with previous data observed in our laboratory, where we have demonstrated that TRAP causes an increase in the percentage of atretic follicles in gonadotropin-treated rat ovaries [31].

Another characteristic of OHSS is the presence of a massive bilateral cystic ovarian enlargement. The ovaries from OHSS rats have a significant degree of stromal edema with multiple hemorrhagic follicular and theca-lutein cysts [52]. In this study, we observed a high percentage of cystic structures in sections of ovaries from OHSS rats, several of which were bleeding. In contrast, we detected a lower number of cysts in the TRAP-treated group, suggesting that TRAP treatment improves follicular development, leading a larger number of follicles to undergo atresia rather than to form cystic structures. The decrease in ovarian weight and serum P_4 and E_2 concentration observed after VEGF inhibition strengthens this conclusion, since atretic follicles are smaller and produce lower levels of E_2 and P_4 than cystic structures.

CL are highly vascularized organs whose vascular density exceeds that of most tumors [53]. Inhibition of VEGF activity can affect the formation and function of CL by preventing angiogenesis. VEGF inhibition decreased the periendothelial cell area in luteal tissues compared to the OHSS group without treatment. This suggests that TRAP caused a decrease in the number of endothelial cells, and thus, a decrease in the luteal vasculature that likely led to a reduction in serum P_4 concentrations in the OHSS model. These results are consistent with those observed by Fraser et al., who showed that the *in vivo* inhibition of VEGF decreases P_4 concentrations in non-human primates [54].

As VEGF acts through its receptor KDR [29,55], we next evaluated the expression and phosphorylation of this receptor in our OHSS model. TRAP treatment affects not only KDR receptor expression but also its activation due to the absence of functional VEGF. Wulff et al. demonstrated a downregulation of KDR and Flt-1 receptors after TRAP treatment in the ovary from marmoset monkey [56]. We observed that the changes in the KDR receptor after TRAP treatment may be due to a low ovarian expression of

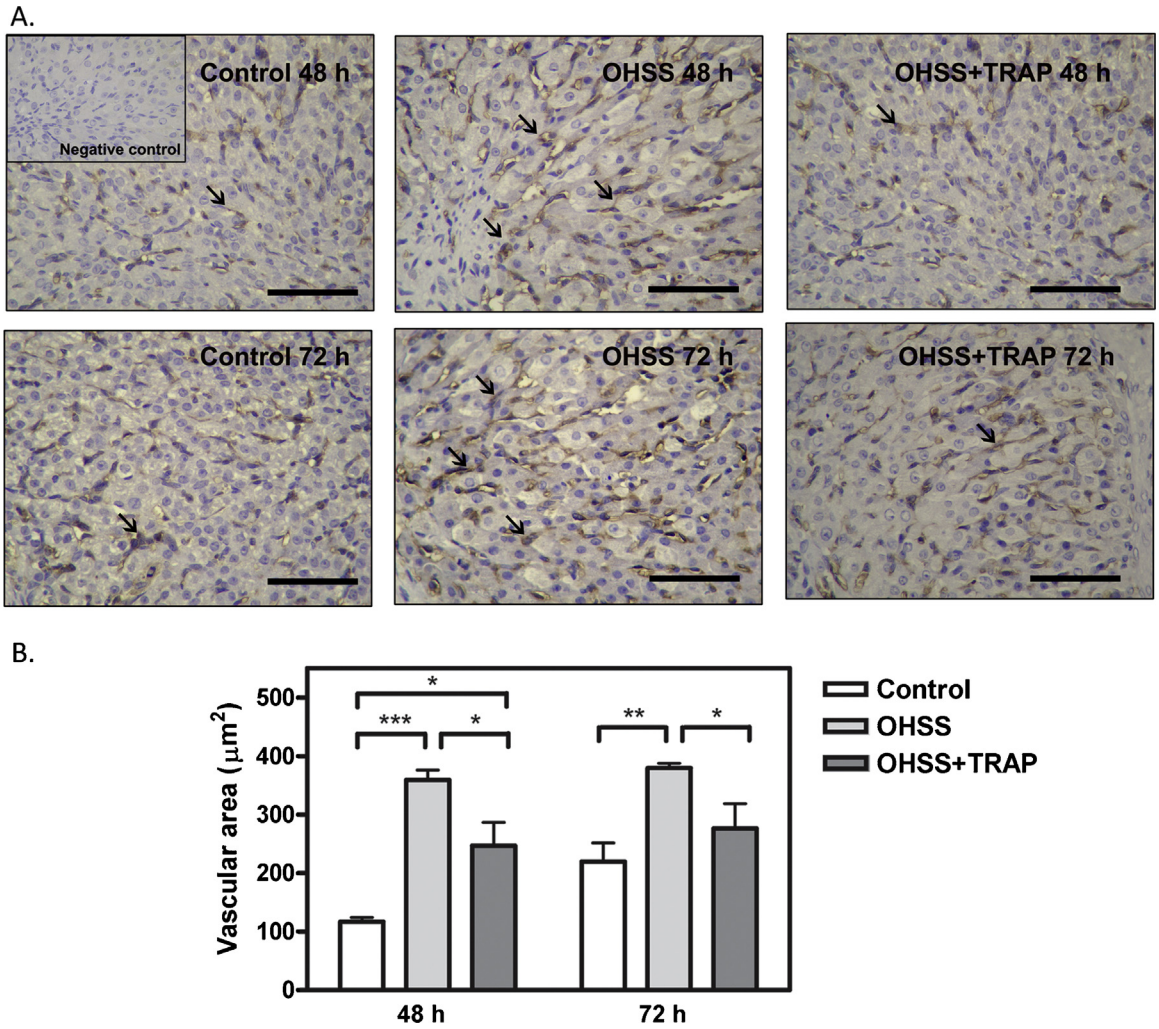

Fig. 4. Effects of TRAP treatment on vascular density measured by the area occupied by BS-1 positive endothelial cells. (A) Photographs show representative fields of ovarian sections stained for lectin BS-1 in the six experimental groups. Arrows show positive staining for lectin in endothelial cells. Original magnification 400X. Scale bars represent 50 μm. The inset shows the negative control. (B) Quantification of vascular area in CL as assessed by lectin BS-I histochemistry. Data are expressed as mean ± SEM. Six sections per CL, ten CL per ovary and six ovaries per group were analyzed ($n = 6$ ovaries/group, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

this receptor. Besides, VEGF seems to regulate the expression of its own receptor in the ovary from our OHSS model. This up-regulation has been described in other tissues [57,58].

VEGF has been shown to activate the PI3K/AKT pathway in different cell types, such as ovarian cells, endothelial cells, and smooth muscle cells, through the KDR receptor [43,59,60]. In this study, we showed an increase in the phosphorylation of AKT in our OHSS model. TRAP treatment decreased AKT phosphorylation. All these results provide evidence that TRAP effectively affects the VEGF system in this OHSS model.

Intercellular junctions mediate adhesion, communication and permeability between steroidogenic and endothelial cells [25,61]. VEGF signaling is thought to mediate the redistribution of TJ proteins and the loss of the endothelial cell barrier architecture [27]. Based on this information, we analyzed the expression of claudin-5 and occludin, two of the main components of TJ proteins in the rat OHSS model [62–65]. We showed that claudin-5 and occludin expression is down-regulated, which would in turn lead to increased vascular permeability as a result of altered barrier architecture. TRAP treatment prevented the decrease in these TJ protein levels. These findings suggest that TRAP treatment is able to restore the levels of claudin-5 and occludin, contributing to the decrease in ovarian vascular permeability observed in this syndrome. Consistent with these results, in non-human primate luteal vasculature, Rodewald et al. demonstrated that after VEGF inhibition, increased claudin-5 and occludin expression may result in sealing of the intercellular space, which may affect paracellular transport and, in turn, prevent hormone precursors from reaching the luteal cells [20]. It is important to mention that the possible involvement of adherens junction proteins, such as VE-cadherin, in the regulation of vascular permeability cannot be ruled out. In this regard, Herr et al. showed that knockdown of VE-cadherin and the subsequent downregulation of claudin-5 causes an increase in the permeability of HUVECs [66]. Besides, the use of antibodies targeted to major endothelial TJ proteins such as claudin-5 and occludin could provide new insights into the prevention and treatment of OHSS.

In summary, this study shows for the first time that VEGF inhibition exerts diverse actions in the ovary from an OHSS rat model. TRAP decreased serum E_2 and P_4 concentrations the percentage of CL and cystic structures, cell proliferation, endothelial cell area and phosphorylation and expression of KDR in ovaries from OHSS rats, whereas it increased luteal apoptosis, claudin-5 and occludin protein levels in OHSS rats.

In conclusion, the results described in the present study indicate that the treatment with TRAP may prevent the early onset of OHSS and decrease its severity.

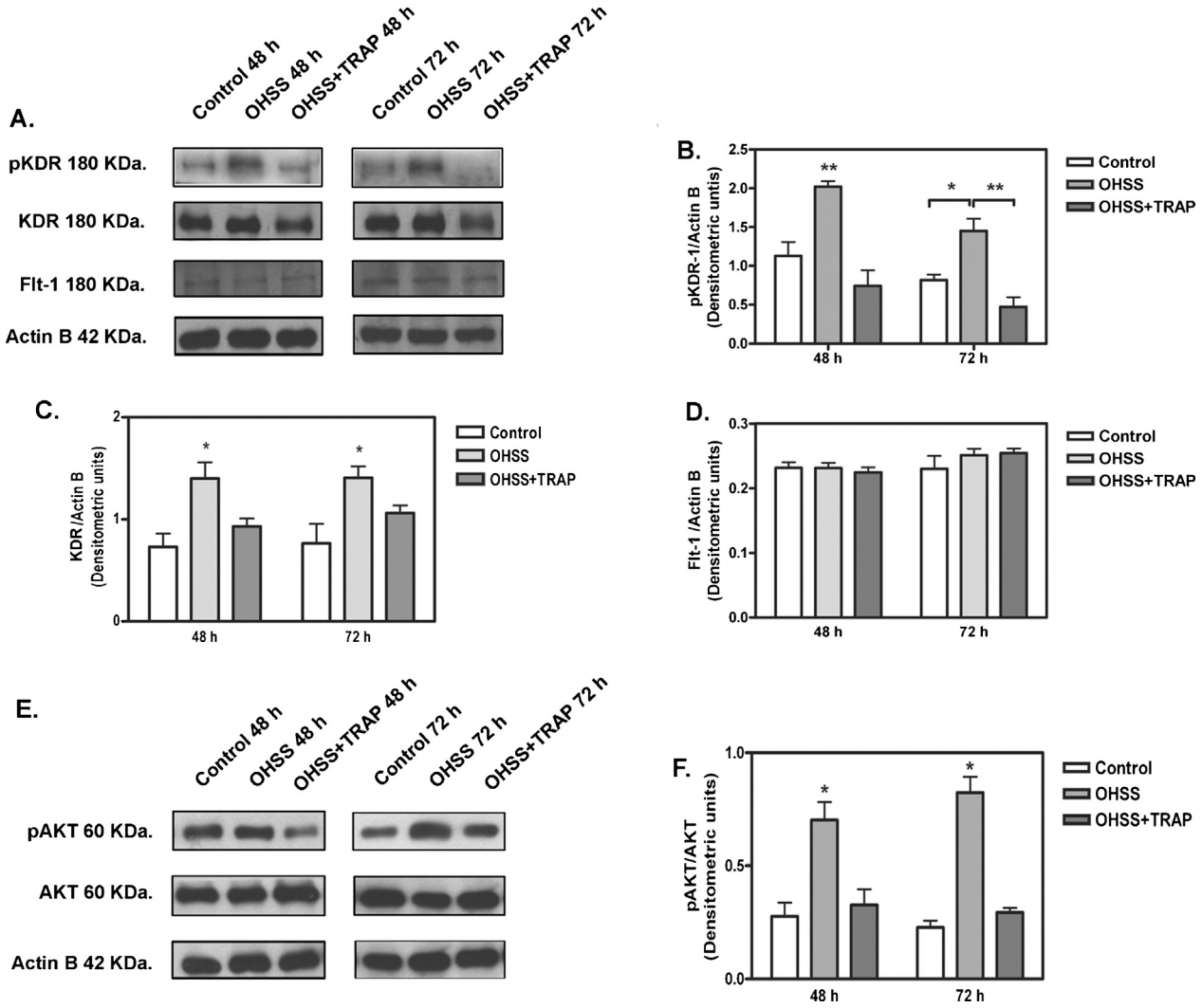

Fig. 5. Effect of TRAP tratment on VEGF receptors and pAKT/AKT expression in ovaries from a rat OHSS model. (A) Representative immunoblots of pKDR, KDR and Flt-1. Densitometric quantification of (B) pKDR, (C) KDR and D) Flt-1. (E) Representative immunoblots of pAKT and AKT. (F) Densitometric quantification of the pAKT/AKT ratio. Optical density is expressed as arbitrary units ± SEM normalized to B actin. $n = 6$/group, pKDR: control 48 h vs. OHSS 48 h, $^{*}p < 0.01$; OHSS 48 h vs. OHSS + TRAP 48 h, $^{*}p < 0.01$. KDR: control 48 h vs. OHSS 48 h, $^{*}p < 0.05$; OHSS 48 h vs. OHSS + TRAP 48 h, $^{*}p < 0.05$; control 72 h vs. OHSS 72 h, $^{*}p < 0.05$; OHSS 72 h vs. OHSS + TRAP 72 h, $^{*}p < 0.05$). pAKT/AKT: control 48 h vs. OHSS 48 h, $^{*}p < 0.05$; OHSS 48 h vs. OHSS + TRAP 48 h, $^{*}p < 0.05$; control 72 h vs. OHSS 72 h, $^{*}p < 0.05$; OHSS 72 h vs. OHSS + TRAP 72 h, $^{*}p < 0.05$).

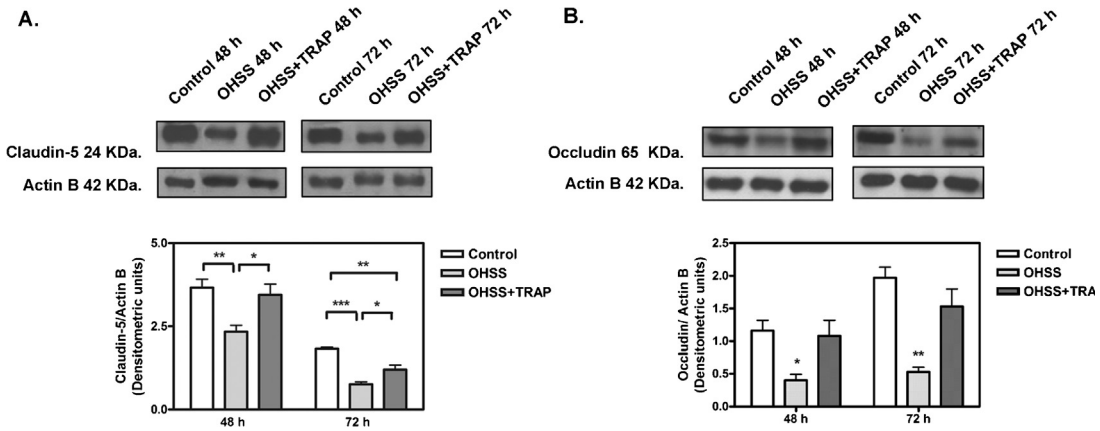

Fig. 6. Effect of TRAP treatment on the expression of tight junction proteins in ovaries from a rat OHSS model. (A) Representative immunoblots of Claudin-5 expression and densitometric quantification. (B) Representative immunoblots of Occludin expression and densitometric quantification. Optical density is expressed as arbitrary units ± SEM normalized to B actin. $n = 6$/group, Claudin-5: control 48 h vs. OHSS 48 h, $^{**}p < 0.01$; OHSS 48 h vs. OHSS + TRAP 48 h, $^{*}p < 0.05$; control 72 h vs. OHSS 72 h, $^{***}p < 0.001$; OHSS 72 h vs. OHSS + TRAP 72 h, $^{*}p < 0.05$. Occludin: control 48 h vs. OHSS 48 h, $^{*}p < 0.05$; OHSS 48 h vs. OHSS + TRAP 48 h, $^{*}p < 0.05$; control 72 h vs. OHSS 72 h, $^{**}p < 0.01$; OHSS 72 h vs. OHSS + TRAP 72 h, $^{*}p < 0.01$.

Therefore, the use of antiangiogenic compounds may contribute to the development of new therapeutic strategies for this pathology.

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