ORIGINAL ARTICLE

https://doi.org/10.1590/1806-9282.20230789

Aflibercept suppresses ovarian hyperstimulation syndrome: an experimental study in rats

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SUMMARY

OBJECTIVE: In this study, we aimed to determine the impact of the antiangiogenic medications, namely, aflibercept and cabergoline in the prevention and treatment of ovarian hyperstimulation syndrome in a rat model.

METHODS: A total of 36 female Wistar rats were randomly allocated to one of the five groups, including disease-free and ovarian hyperstimulation syndrome controls: Group no OHSS (control, n=6) received saline only intraperitoneally (i.p.); group just OHSS (ovarian hyperstimulation syndrome only, n=6) received 10 IU pregnant mare serum gonadotropin and 30 IU human chorionic gonadotropin subcutaneously to produce ovarian hyperstimulation syndrome; group cabergoline+OHSS (cabergoline+ovarian hyperstimulation syndrome, n=8) received 100 μg/kg oral cabergoline; group aflibercept (12.5 mg/kg)+OHSS (aflibercept+ovarian hyperstimulation syndrome, n=8) received 12.5 mg/kg i.p. aflibercept ; and group aflibercept (25 mg/kg)+OHSS (aflibercept+ovarian hyperstimulation syndrome, n=8) received 25 mg/kg i.p. aflibercept. The groups were compared for ovarian weight, immunohistochemical vascular endothelial growth factor expression, spectrophotometric vascular permeability evaluated with methylene blue solution in peritoneal lavage, and body weight growth.

RESULTS: Vascular endothelial growth factor immunoexpression was substantially greater in the just OHSS group (22.00±10.20%) than in the aflibercept (12.5 mg/kg)+OHSS (7.87±6.13%) and aflibercept (25 mg/kg)+OHSS (5.63±4.53%) groups (p=0.008 and p=0.005, respectively). Post-hoc tests indicated that cabergoline, 12.5 mg/kg aflibercept, and 25 mg/kg aflibercept decreased vascular permeability compared to the untreated ovarian hyperstimulation syndrome group (p=0.003, p=0.003, and p=0.001, respectively). JOH group had the heaviest ovaries, whereas aflibercept (25 mg/kg)+OHSS group had the lightest. In terms of body weight gain, cabergoline+OHSS group was substantially greater than the aflibercept (12.5 mg/kg)+OHSS and aflibercept (25 mg/kg)+OHSS groups (p=0.006 and p=0.007, respectively).

CONCLUSION: Aflibercept, an antiangiogenic medication, decreased ovarian hyperstimulation syndrome by lowering the vascular permeability and vascular endothelial growth factor expression.

KEYWORDS: Aflibercept. Cabergoline. Ovarian hyperstimulation syndrome. Vascular endothelial growth factors.

INTRODUCTION

Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic result of ovarian stimulation that can be potentially fatal. It is characterized by cystic growth of the ovaries and rapid transudation of protein-rich fluid from the vessels into the abdominal cavity. The extra fluid leads to weight gain, abdominal distension, and intravascular depletion. In severe OHSS, this protein-rich fluid may also be detected in the pleural and pericardial cavities[1].

Human chorionic gonadotropin (hCG) used for triggering ovulation in ovulation induction (OI) and IVF cycles is considered to have a pivotal role in the development of this life-threatening disease. After exposure to hCG, there is a strong luteinization of granulosa cells in the corpus luteum of the hyperstimulated ovaries, resulting in OHSS. Vasoactive substances such as vascular endothelial growth factor (VEGF) are produced, which increases vascular permeability, and as a result of the fluid scape from the vessels into the third space, signs and symptoms are related to the consequential edema, ascites, pleural and pleural effusion, and hemoconcentration development[2]. VEGF and VEGF receptor (VEGFR) polymorphisms are reported to be related to the formation of OHSS. IVF recipients who develop OHSS are known to produce less soluble VEGFR1 than OHSS-free recipients, and VEGF concentration has been shown to be directly proportional to the severity of OHSS. Consequently, the pathophysiology of

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Conflicts of interest: the authors declare there is no conflicts of interest. Funding: The study was funded by the researchers. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.
Received on July 26, 2023. Accepted on August 03, 2023.

OHSS relies on the VEGF/VEGFR system, which has a significant impact on vascular permeability[2,3].

Currently, there are different strategies for prevention of OHSS in clinical practice: primarily, lowering the hCG dose at trigger, using gonadotropin agonist (GnRH-A) for triggering, use of progesterone as luteal phase support rather than hCG, embryo cryopreservation as opposed to immediate fresh embryo transfer, a "freeze-all" technique for patients at high risk of OHSS, and canceling the treatment cycle when abnormally high number of follicles grow and/or there is a dramatic estradiol rise[4]. In addition, cabergoline, an ergot-derived molecule known to be a potent dopamine receptor agonist (DA), is commonly administered by reproductive physicians to treat OHSS[5].

Among VEGFRs, only VEGFR1 and VEGFR2 have been shown to be directly involved in VEGF binding. On the contrary, IVF recipients who develop OHSS are known to produce less soluble VEGFR1 than OHSS-free recipients, and VEGF concentration has been shown to be directly proportional to OHSS. Consequently, the pathophysiology of OHSS relies on this VEGF/VEGFR system, which has a significant impact on vascular permeability[3].

Cabergoline, a D2 receptor agonist, has been shown to have potent inhibitory effects on VEGF by altering both peptide synthesis and secretion[6]. Aflibercept is a recombinant receptor fusion protein that inhibits angiogenesis by combining the second and third domains of human VEGFR1 and VEGFR2[7].

Based on the angiogenic pathophysiology of OHSS, antiangiogenic drugs have a potential in the treatment of this syndrome. We report an experiment in rats comparing aflibercept, whose effect on OHSS has not yet been studied, with cabergoline, which has been extensively studied.

METHODS

Animals and ethical approval

A total of 36 female Wistar albino rats were studied. Notably, 22-day-old rats weighed 35–65 g. Other species, sex, age, weight, mortality during the experiment, and suspected sickness were excluded. Steel cages with a 12-h light-dark cycle held animals in a temperature-controlled room (22±2°C). All animals were free-fed. The Animal Research Ethics Committee of Ankara University approved the Project (16/03/2022-06-32-6-58). The rats were obtained from the Ankara University Animal Laboratory, where the animals were cared for and the experiment was conducted. In all animal experiments, the rules of the Human Care Committee for animal experiments were completely followed.

Experimental concept

The experiment was designed in a randomized controlled manner with five different groups. Randomization was performed using the Microsoft Excel function RAND.

1. The NOH group (no OHSS, control, n=6) received only 0.1 mL of saline intraperitoneally (i.p.) daily for 5 days.
2. To induce OHSS in the JOH group (OHSS only, n=6), 10 IU of pregnant mare serum gonadotropin (PMSG) (Folligon®-Intervet; Schering-Plough Animal Health, Pune, India) was administered subcutaneously (s.c.) for 4 days, followed by 30 IU of hCG (Chorulon®-Intervet, Boxmeer, The Netherlands) on the 5th day.
3. The COH group (cabergoline+OHSS, n=8) received cabergoline (100 µg/kg, Dostinex®-Pharmacia SpA, Ascoli Piceno, Italy) dissolved in 1 mL of tap water, via oral gavage (0.8 mm´45 mm curved delivery cannula), 2 h before each of the 5-day injections required for OHSS stimulation (first 4 days 10 IU PMSG, 5th day 30 IU hCG).
4. The aOH (aflibercept, 12.5 mg/kg+OHSS, n=8) group received i.p. aflibercept (Eylea®; Regeneron, NY, USA) at a dose of 12.5 mg/kg once, 2 h before the first PMSG injection for OHSS stimulation.
5. The AOH group (aflibercept, 25 mg/kg+OHSS, n=8) received aflibercept at a dose of 25 mg/kg once in the same manner. The literature was used to develop the procedure for the OHSS model and the cabergoline doses[1]. The dosage of aflibercept was determined on the basis of previous studies and taking into account the opinion of the pharmacologist[8].

Surgical procedure and vascular permeability determination

All rats (28 days old) were weighed 48 h after hCG administration on the 5th day. The 7-day body weight gains (BWG) were calculated and noted. Then, rats aged 28 days, i.p. 90 mg/kg ketamine hydrochloride (Ketalar®, Eczacıbaşı Warner-Lambert pharmaceutical industry, Levent/Istanbul) and i.p. 10 mg/kg xylazine (Rompun-Bayer®, Şişli/Istanbul) were administered to induce anesthesia. Antisepsis with 10% povidone-iodine solution was performed before surgery. Immediately after confirmation of the depth of anesthesia by skin pinch reaction, 0.2 mL of methylene blue solution (methylene blue solution 1% w/v, Gunduz Chemical®, Umraniye/Istanbul) was administered to the rats through the tail vein via an insulin injector. After a 4 cm vertical incision, the peritoneal cavity was filled with 5 mL of 0.9% NaCl solution. After 30 min, the peritoneal cleansing solutions were extracted using a fertilization

catheter with constant shaking and without tissue damage. These fluids collected for vascular permeability determination were placed in tubes containing 0.05 mL of 0.1 N NaOH and centrifuged (900´g, 30 min).

Methylene blue concentration (MBC) was determined using a spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) at 665 nm. The concentration of extravasated dye in the recovered fluid was quantified as micrograms (µg) per 100 g of body weight. The surgical procedure was continued by carefully scraping and excising the bilateral ovaries from the surrounding tissue. The removed bilateral ovaries were weighed (BOW) jointly on a sensitive scale (Radwag© PS 0.6.R2) and then placed in containers that have 10% formolin. Finally, all rats were sacrificed by cervical dislocation while under anesthesia.

Immunohistochemical vascular endothelial growth factor expression

Ovarian tissue samples were embedded in paraffin. Notably, 5 µm sections were made of all paraffin-embedded tissues on the microtome (Leica-RM225 - Thermo HM3555 - Thermo scientific). The Ventana BenchMark XT system (Ventana Medical Systems, Roche, Basel, Switzerland) was used to automatically perform immunohistochemical staining. The universal 3,3'-diaminobenzidine detection kit ultraview (DAB) (Ventana®) was used for automated immunohistochemistry equipment. A positive control for the primary VEGF antibody (Flt-1/VEGFR1, 0.1 mL concentrate 1:501:200 antibody, GenomeME, Richmond BC, Canada) was prepared on each slice. The staining of the cytoplasm was assessed. The number of VEGF-positive cells was determined by counting at least 100 granulosa cells per 10 tissue slices at 100× magnification (Nikon® ECLIPSE 80i, Japan), and cross sections were photographed (NIS -Elements D Ver5.02.03 for 64bit edition software). A pathologist conducted these procedures blindly in a pathology clinic.

Statistical analysis

GPower 3.1 determined the research sample size. Shapiro-Wilk test was used to determine data normality. Continuous variables have mean±standard deviation. One-way analysis of variance (ANOVA) was performed for normal variables and Kruskal-Wallis test was performed for non-normal variables to compare groups. Post-hoc analyses used Bonferroni correction. SPSS.25 was used for analysis, and $p<0.05$ was considered significant.

RESULTS

Body weight development was significantly different across groups, while total bilateral ovary weight was not (Table 1).

The Kruskal-Wallis test revealed a statistically significant difference between groups based on the measured concentrations of methylene blue (µg/100 g) to determine vascular permeability. Post-hoc tests showed that cabergoline (2.85±2.79 µg), 12.5 mg/kg aflibercept (1.93±2.63 µg), and 25 mg/kg aflibercept (0.94±0.89 µg) treatments significantly decreased vascular permeability compared to the untreated OHSS group (9.88±2.73 µg) (p=0.003, p=0.003, p=0.001, respectively). The values and significance levels are shown in Table 1. Figure 1 shows a graphical representation of the MBCs.

The strongest staining in granulosa cells was observed in the untreated OHSS group (Figure 2B). Virtually, no VEGF staining was observed in the control group (Figure 2A). The COH, aOH, and AOH groups showed significantly lower VEGF expression (Figures 2C–E). The Kruskal-Wallis test showed that there was a significant difference in the percentage of VEGF immunoexpression between the groups (p=0.004). According to the post-hoc tests, the percentage of VEGF immunoexpression was significantly higher in the JOH group (22.00±10.20%) than in the aOH group (7.87±6.13%) and the AOH group (5.63±4.53%) (p=0.008 and p=0.005,

Table 1. Comparison of group parameters for body weight gain, bilateral ovarian weight, vascular permeability, and vascular endothelial growth factor expression.

Groups	NOH control (n=6)	JOH OHSS (n=6)	COH OHSS+cabergoline (n=8)	aOH OHSS+aflibercept^{x} (n=8)	AOH OHSS+aflibercept^{y} (n=8)
BWG* (g)	22.00±5.44	23.50±3.51	25.75±2.71	17.13±6.79*	17.25±3.01**
BOW* (mg)	53.50±15.93	66.00±7.51	57.00±12.99	51.12±8.49	48.25±13.78
MBC** (µg/100 g)	2.40±2.33	9.88±2.73^{a}	2.85±2.79^{b}	1.93±2.63^{c}	0.94±0.89^{d}
VEGF** (%)	6.50±3.41	22.00±10.20	9.88±8.59	7.87±6.13^{e}	5.63±4.53^{f}

OHSS: ovarian hyperstimulation syndrome; BWG: body weight gain; BOW: bilateral ovarian weight; MBC: methylene blue concentration; VEGF: percentage of VEGF-positive cells. It was determined by counting at least 100 granulosa cells per 10 tissue slices at 100´ magnification. ^{x}12.5 mg/kg, ^{y}25 mg/kg, *One-way ANOVA analysis, **Kruskal-Wallis test. *p=0.006 COH group and aOH group were compared. **p=0.007 COH group and AOH group were compared. ^{a}p=0.004 JOH group and NOH group were compared. ^{b}p=0.003 JOH group and COH group were compared. ^{c}p=0.003 JOH group and aOH group were compared. ^{d}p=0.001 JOH group and AOH group were compared. ^{e}p=0.008 JOH group and aOH group were compared. ^{f}p=0.005 JOH group and AOH group were compared.

respectively). However, there was no significant difference in the percentage of VEGF immunoexpression between the other groups (Table 1 and Figure 3).

DISCUSSION

This study focused specifically on vascular permeability and VEGF expression. In this study, the antiangiogenic drug aflibercept was used for the first time as a therapy for OHSS. Since this is the first time the drug has been administered in OHSS, the study was organized as an animal experiment. Aflibercept has a preventative effect on two of the most important elements of OHSS: inhibition of vascular permeability and reduction of VEGF expression[1,9].

Daily monitoring of patients' body weight and waist circumference is recommended in the guidelines for the management of OHSS[10]. The importance of measuring the waist circumference of rats in OHSS is not known; therefore, we examined body weight before and after OHSS and compared the amount of weight gain in the different groups. This discrepancy between the final and initial body weight was also used to confirm OHSS induction. The results showed that the aflibercept-treated group gained less weight than the cabergoline-treated group (Table 1). This indicates that aflibercept inhibits OHSS; however, it should be noted that the slower weight gain could be a side effect of aflibercept, as we did not observe similar results in the cabergoline group and 22-day-old rats are normally in a rapid growth phase. In a previous

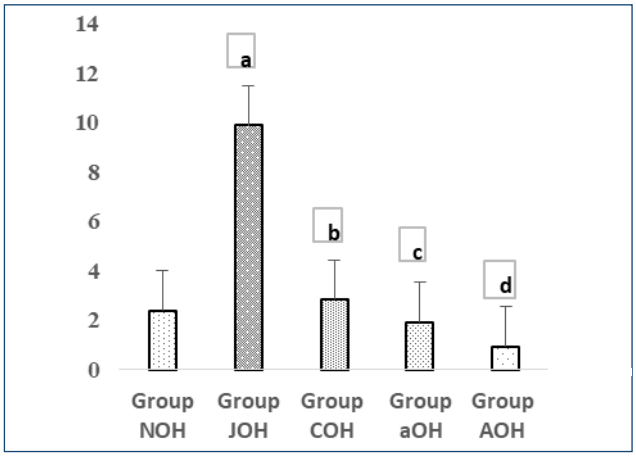

Figure 1. Comparison of vascular permeability between groups. NOH: no OHSS, control; JOH: just OHSS, no treatment; COH: cabergoline+OHSS; aOH: aflibercept (12.5 mg/kg)+OHSS; and AOH: aflibercept (25 mg/kg)+OHSS. Comparison between groups was performed using Kruskal-Wallis test, and in case of significant difference post-hoc analyses with Bonferroni correction were performed. $p<0.05$ were considered significant. [a]$p=0.004$ JOH group and NOH group were compared. [b]$p=0.003$ JOH group and COH group were compared. [c]$p=0.003$ JOH group and aOH group were compared. [d]$p=0.001$ JOH group and AOH group were compared.

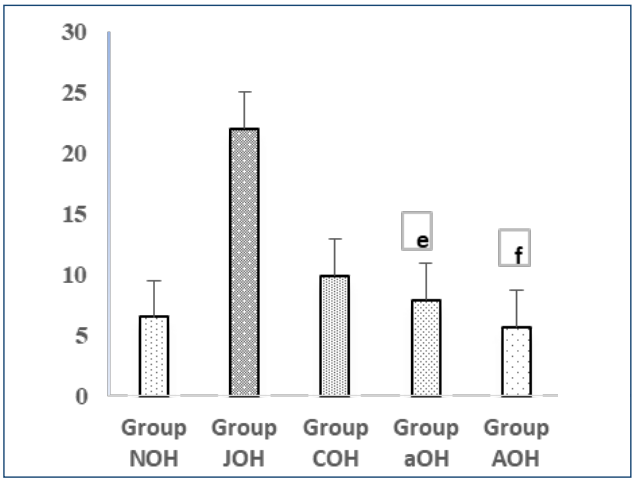

Figure 3. Comparison of vascular endothelial growth factor expression between groups. NOH: no OHSS, control; JOH: just OHSS, no treatment; COH: cabergoline+OHSS; aOH: aflibercept (12.5 mg/kg)+OHSS; AOH: aflibercept (25 mg/kg)+OHSS. Comparison between groups was performed using Kruskal-Wallis test, and in case of significant difference post-hoc analyses with Bonferroni correction were performed. $p<0.05$ were considered significant. [e]$p=0.008$ JOH group and aOH group were compared. [f]$p=0.005$ JOH group and AOH group were compared.

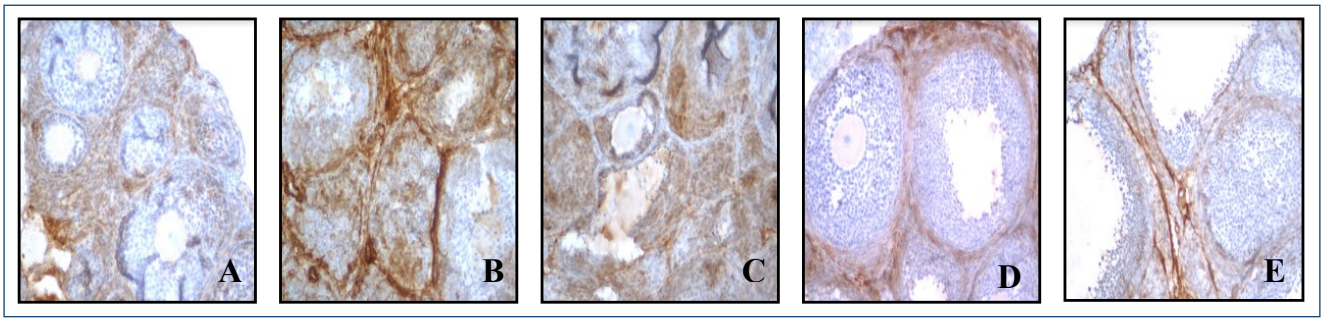

Figure 2. Vascular endothelial growth factor immunoexpression in the ovaries. (A) Control group, less staining; (B) increased expression of vascular endothelial growth factor in granulosa cells in the ovarian hyperstimulation syndrome group; (C) decreased vascular endothelial growth factor expression in the cabergoline group; (D) more decreased vascular endothelial growth factor expression in the 12.5 mg/kg aflibercept group; and (E) much more decreased vascular endothelial growth factor expression in the 25 mg/kg aflibercept group. Magnification 200×.

study, aflibercept was shown to decrease body weight gain[11]. Our study showed that the OHSS group had heavier ovaries than the control group. In the aflibercept-treated groups, ovarian weight decreased proportionally with increasing dose. Although these results are not statistically significant, they are consistent with those of previous studies[1,9].

Ovarian hyperstimulation syndrome's main pathology is capillary permeability. In vulnerable individuals, increased VEGF release in luteinized granulosa cells of the ovary causes symptoms to initiate and develop[12]. Therefore, antiangiogenic drugs are capable of treating OHSS. There are various experiments in the literature for the mechanism of inhibition of angiogenesis: Şanlı et al., focused on oxidative stress sensitive transient receptor potential melastatin 2 (TRPM2) channels that can induce angiogenesis in OHSS. Pala et al., focused on the antiproliferative effect of tamoxifen by blocking the mitogenic effect of estrogen. Zhai et al., focused on the suppression of VEGF expression through the Kisspeptin / KISS1R (KISS1 receptor) system[2,13,14]. In our study, OHSS rats showed higher VEGF levels. Aflibercept reduced VEGF expression and was more effective in treatment than cabergoline.

Early administration of cabergoline is a safe and potentially more effective method for prophylaxis of OHSS in high-risk settings[15]. Our study does not provide information on the clinical safety of aflibercept in OHSS. However, aflibercept is now preferred as first-line therapy for diabetic retinopathy because the risk of complications is lower than with other anti-VEGF drugs[16]. According to ophthalmologists, bevacizumab, another anti-VEGF drug, has a lower success rate in treating retinopathy than aflibercept[17]. It has been shown that another anti-VEGF drug, bevacizumab, was better in treating OHSS than cabergoline when the peritoneal VEGF levels were measured in both groups[18]. On the contrary, we investigated the permeability of peritoneal fluid with methylene blue solution. Our results show that aflibercept is very effective in both inhibiting vascular permeability and reducing VEGF expression, two of the most important indicators of OHSS. The fact that we formed groups that received two different amounts of therapy can be considered a means of validating the results.

Our study has some limitations. Due to its restricted characteristics, this study cannot compare side effects. Ultimately, this is an animal study that is not transferable to humans. Systemic anti-VEGF medicines raise arterial blood pressure, thromboembolic events, left ventricular dilatation, and contractile dysfunction[19]. On the contrary, cabergoline can cause serious side effects such as respiratory problems, complications with heart valves, and risk of addictive behavior, in addition to headache, dizziness, nausea, and constipation[20].Treatments accompanied the OHSS induction. Hence, this study should be viewed more as a preventative measure. Therefore, the acceptance of aflibercept as a treatment option for OHSS depends on further animal studies and clinical trials.

CONCLUSION

Our pioneering study shows that aflibercept, an antiangiogenic drug, effectively reduces OHSS by decreasing vascular permeability and VEGF expression. This study shows that the efficacy of aflibercept in OHSS is significantly more prominent than that of cabergoline.

DATA SHARING STATEMENT

All data from this research, which was planned as an animal study, will be made available upon request. The authors state that the information may be published in the data sharing statement. Data include Excel and/or SPSS version of results obtained for statistical analysis, hematoxylin-eosin stained preparations, immunohistochemistry stained preparations, and any other information obtained.

ETHICAL APPROVAL

The Ankara University Animal Research Ethics Committee approved the project (meeting date: 16/03/2022, meeting number: 2022-06, file number: 2022-32, and decision number: 2022-6-58). The rats were obtained from the animal laboratory of Ankara University, where the animals were cared for and the experiment was conducted. In all animal experiments, the rules of the Human Care Committee for Animal Experiments were completely followed.

AUTHORS' CONTRIBUTIONS

ÇA: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Visualization, Writing – original draft. **BD:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – review & editing. S**YE:** Data curation, Formal Analysis, Funding acquisition, Resources, Software, Validation, Visualization, Writing – original draft. **FA:** Formal Analysis, Funding acquisition, Methodology, Software, Validation, Visualization, Writing – original draft.

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