The evaluation of honey bee venom potential therapeutic value on gonadotropin-induced ovarian hyperstimulation syndrome model rats

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Research Article

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Abstract

Ovarian hyperstimulation syndrome (OHSS) mostly occurs in undergoing gonadotropin therapy women sensitive to endogenous gonadotropins. Honey bee venom (HBV) contains biologically active components that has pharmaceutical properties. This study was designed to assess the possibility of HBV application as an anti-inflammatory and anti-angiopathy therapeutic agent to suppress main inflammatory mediators in OHSS. OHSS induced by Pregnant Mare's Serum Gonadotropin) PMSG) and hCG intraperitoneal in immature female Wistar rats. OHSS induction followed by intraperitoneal injection of HBV and metformin for 10 consecutive days; At the end of the treatment period, metabolic parameters as well as ovarian expression of IL-6, COX2, and VEGF proteins were evaluated. This data showed that the OHSS+HBV group had a significant reduction in peritoneal and ovarian vascular permeability compared to the OHSS group. The ovarian expression of COX2, IL-6 and VEGF proteins in group OHSS+HBV. The results of this study showed that HBV can affect the inflammatory feature of OHSS by reducing the angiogenesis factors.

1. Introduction

Ovarian hyperstimulation syndrome (OHSS) is a probable consequence of assisted reproductive techniques and a systemic disease resulting from vasoactive products released by hyperstimulated ovaries under transfusion of human chorionic gonadotropin (hCG)(Castillo et al. 2020). The incidence of low and moderate OHSS is estimated to be between 3 % and 6% recepectively, while the severe OHSS may occur in 0.1 %–3% of all reproductive cycles (Diao et al. 2007) . The primary risk factors for OHSS include escalation of response to ovarian stimulation at a young age, a history of increased response to gonadotropins, previous OHSS, and polycystic ovary syndrome. Secondary risk factors include ovarian response parameters such as the increase in serum estradiol (E2), the size and number of follicles, and the number of released oocytes (Braam et al. 2020; Farkas et al. 2020; Blumenfeld 2018).

In response to hormonal stimulation, inflammatory mediators such as interleukins (ILs), tumor necrosis factor-α (TNF-α), and vascular mediators like interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) secreted by the ovaries , these the factors are involved in increasing membrane permeability (Soares et al. 2008). More than 90% of patients complained of dyspnea and ascites due to ovarian enlargement, severe rupture of follicles and increased intra-abdominal pressure (Timmons et al. 2019).

In OHSS, phenomena of oxidative stress, angiogenesis, and inflammation by intensifying the effects of each other cause the symptoms of this syndrome during the sensitive life of the mother and the fetus (Diao et al. 2007; Rizk et al. 1985). Expression and secretion of IL-6 that mediates leukocytosis and vascular permeability, increases expression of COX-2 due to nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling pathway in the pathogenesis of hepatic dysfunction. Increased levels of COX-2 play confirmed a role in a state of inflammation, oxidative stress and reproductive disorders in women (Soares et al. 2008; Budak et al. 2012; Quintana et al. 2008; Latifeh Karimzadeh et al. 2013).
Complications of gonadotropin in these groups include reduced implantation rate, poor quality of oocytes, ascites and severe leukocytosis and kidney damage in the final stages (Diao et al. 2007; Oršolić 2012). For providing safe pregnancy for mother and fetus, it is expected to reduce the symptoms of this syndrome by reducing the critical level of angiogenesis, oxidative stress, and inflammation.

Bee venom contains 18 active components that contain various peptides including melittin, apamin, and adipoline, bioactive amino acids such as histamine and epinephrine. HBV combination of pharmacological and biochemical active agents has been used in various studies as an analgesic, anti-inflammatory, and anti-tumor agent (Mousavi et al. 2012; Oršolić 2012; L. Karimzadeh et al. 2012; Silva et al. 2015; Tender et al. 2032; Dantas et al. 2014; Zhang et al. 2018; Wehbe et al. 2019).

Given that HBV has been effective in oocyte maturation and ameliorating the symptoms of polycystic ovary syndrome in rats, it is expected to be also helpful in reducing OHSS inflammation (Soares et al. 2008). The purpose of this study was to evaluate the changes in the levels of proteins involved in inflammation and to reduce the metabolic and physiological symptoms of rats with OHSS under HBV therapy (Apitherapy) as an experimental treatment.

2. Materials And Methods

2.1. Drugs and reagents

General chemicals of analytical grade were obtained from Sigma (UK) and Merck (Germany). Pregnant mare's serum gonadotropin (PMSG) (Pregnecol®) was purchased from bionic (Australia/Asia). The HCG was obtained from LG life sciences (Korea). Ketamine and xylazine were purchased from Alsafean (Holland). Primary VEGF and COX-2 antibody (Dako, Denmark), and IL6 antibody (HPA005825 Anti-SERPINF1, Atlas Antibodies, Sweden) were purchased. IL-6 ELISA kit (rat IL-6 platinum ELISA®, Bender Medsystems, Austria), and CRP ELISA kit (Millipore's MILLIPLEX® MAP Rat/Mouse CRP Single Plex USA) were used. Lipid profile (CHODPAP/endpoint method using reagents supplied by the biochemistry company), were used. MDA and LDH release was tested by commercial kits (ZiestChem Diagnostics, Iran).

2.2. Experimental animals and ethical aspects

In this experimental study, 48 immature wistar rats (female, weighing 85±10 g, 30-40 days) were used from the animal house of the Kharazmi University, Tehran, Iran. Animals were kept under conditions including 12/12 h light-dark cycle, 23± 3 °C temperature and humidity 47%. Rats were fed a standard diet, allowed free access to food and water. All rats were treated in compliance with the guidelines for the care and use of animals approved by our institutions with (Ethical Code 61694110) in accordance with the principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, D.C).
2.3. Experimental design

Healthy animals were divided into six experimental groups (n=8):

Group 1: Control, Group 2: PBS; received 0.1 ml PBS; (22th – 25th day); IP injection, Group 3: HBV; received 0.5 mg/kg HBV solved in PBS; once a day (4 pm from day 18 to day 27); IP injection, Group 4: OHSS, Group 5: OHSS+HBV; received 0.5 mg/kg HBV; once a day (18th - 27th day); IP injection and Group 6- OHSS+ Met; received 50 µg/kg Metformin, once a day; (18th - 27th day); IP injection

2.4. OHSS induction

To promote follicular development, immature female wistar rats received 10 IU (Subcutaneous Injection) of PMSG. PMSG injection was started from 22th day and on the 26th day of life, rats received 30 IU (Subcutaneous injection) hCG to induce ovulation. The main symptoms of OHSS were hyper latinized ovaries, were increased vascular permeability (VP) 48 h after hCG administration (28th day of life) (Saylan et al. 2010).

2.5. Sample collection

Animals were anesthetized with Ketamine (87 mg/ kg) and xylazine (13 mg/kg) 48 h after the latest injection to measure ovarian and peritoneal vascular permeability (VP). Subsequently, the animals were anathasies via (120 mg/kg) pentobarbital and the blood samples were obtained by cardiopuncture. After centrifugation, serums were stored at -20°C for subsequent analysis of hormones, lipid profile, IL-6, and CRP. In each set of experiments, the livers and ovaries were removed and frozen in liquid nitrogen for MDA, LDH, and VEGF, IL-6, COX-2 and protein analysis, respectively.

2.6. Ovarian and peritoneal vascular permeability

To measure VP, rats were anesthetized with ketamine (100 mg/kg) and kept warm in a thermal blanket to prevent hypothermia. Briefly, a fixed volume (0.2 ml) of 5 mM Evans Blue (EB) dye diluted in distilled water was injected via the femoral vein. Thirty minutes after dye injection, the peritoneal cavity was filled with (5 ml of 0.9% saline; 21 C; pH 6) and massaged for 30 seconds. Subsequently, the fluid was slowly extracted with a vascular catheter to prevent tissue or vessel damage. To prevent any protein interference, peritoneal fluid was recovered in tubes containing NaOH( 0.1N). After centrifugation at 900 g for 12 minutes, EB concentration was measured at 600 nm by spectrophotometer. The level of the extracted dye in the recovered fluid was expressed as microgram of EB per 100 gr body weight (Quintana et al. 2008). Ovaries were removed after blood sampling. Two ovaries from each rat were weighed; the right ovary was frozen at ~80°C, and the left ovary was incubated at 2 ml formamide at 37°C for 24 hours. To evaluate the vesseles permeability in the ovary; EB concentration in the formamide extract of the ovary was also
measured. While the dye concentration in the peritoneal irrigated fluid was presented as (100 μg/g) body weight, EB content in the ovary was presented as ng/mg tissue wet weight.

2.7. Hormone assay

The blood samples were incubated at room temperature for 1 h, and supernatant was collected after centrifugation at 3000 rpm for 20 min. The levels of follicle stimulating hormone (FSH), luteal hormone (LH), testosterone (T), progesterone (P4) and 17-β-estradiol (E2) were evaluated using the ELISA kit according to the manufacturer's instructions.

2.8. Lipid profile and glucose related indexes

Serologic tests were performed for blood samples' serum to determine the changes in serum lipid profile. Fast blood glucose was measured by oxidase reaction glucose oxidase analyzer, beckman, fullerton, CA and expressed in mmol/L. Insulin level was determined by Ultrasensitive (ELISA ALPCO Diagnostics, USA) and expressed in Units. The homeostasis model assessment values for insulin resistance (HOMA-IR) and percent β-cell function (HOMA-% β-cell) were calculated. As described by Matthews, HOMA-IR was calculated using the below formula, HOMA-β was calculated for the insulin secretion ability of pancreatic β-cells in different stages of liver dysfunctions(Y. Chen et al. 2021):

\[
\text{HOMA-IR} = \frac{\text{fasting insulin [μU/ml]} \times \text{fasting glucose [mmol/L]}}{22.5}
\]

\[
\text{HOMA-% β cell} = \frac{20 \times \text{insulin in μU/ml}}{[(\text{glucose in mg/DL}) - 3.5]}
\]

2.9. Enzyme-linked immunosorbent assay (ELISA) for CRP and IL-6

The serological analysis was performed to measure serum IL-6 level alterations. The IL-6 level was determined by an ELISA kit. For this purpose, used a commercially available ELISA kit according to the manufacturer's instructions. The sensitivities of the assay for IL-6 were 12 pg/ml. CRP contents were measured using an ELISA kit. The sensitivities of the assay for CRP were 12 pg/ml.

2.10. Protein isolation from the ovarian and liver samples

Total protein was extracted from ovary individually according to standard methods by using an assay buffer containing (0.2 mg/ml) sodium orthovanadate, a protease, and a phosphatase inhibitor cocktail at the recommended concentrations. Total protein concentration was measured using a Bradford protein assay reagent. Individual lists corresponding to animals in each group (n=10) contributed equally (120 g) to make a pool of 1000 g protein.
2.10.1. Assay of malondialdehyde (MDA) and lactate dehydrogenase (LDH) concentration in the liver

To determine the oxidative stress status, MDA and LDH release was evaluated by commercial kits according to the protocols of the manufacturers. Briefly, the extraction of the liver was added to an enzyme-linked plate along with (60 μL) MDA and LDH test reagents. After dark incubation for 30 min, OD values at 490 nm were measured. MDA and LDH level (in percentage) was calculated as (A Sample–A Blank) (A Control–A Blank) ×100% U/mg protein.

2.10.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and western blot analysis

The proteins were isolated using SDS PAGE and transferred to nitrocellulose membranes during a three-hour period at 300 am and 50 V using a Bio-Rad trans-blot system. Membranes were blocked with 5% nonfat milk powder in TBS-T for 1 h at room temperature. The nitrocellulose membrane was incubated with primary antibody such as IL-6, COX-2, and VEGF at a dilution of 1:1000 for 2 h at room temperature in a humidified chamber and washed three times for 15 min in TBS-T. The membrane was then incubated with horseradish peroxidase-coupled rabbit anti-goat IgG, washed three times for 10 min in TBS-T, then incubated with enhanced chemiluminescence (ECL) reagent for 5 min and exposed to X-ray film for several minutes in a dark room. A probe of β-actin was used as internal control. The levels of protein were compared and analyzed by densitometry studies using Image J software. The density of each band was normalized to the density of the housekeeping protein (β-actin) and control groups.

2.11. Histopathology and immunohistochemistry

The ovarian tissues were stained using immunohistochemical methods to evaluate VEGF, COX-2 and IL-6 expression. The tissue samples were fixed in buffered formalin (7%). According to standard procedure, tissues were embedded in paraffin blocks and cut into 4-µm sections and deparaffinized. One section of ovarian block was stained with hematoxylin and eosin (H&E), and the remaining sections were stained with VEGF, COX-2 and IL6 antibodies using an immunohistochemistry dakocytomation autostainer kit. Local expression of VEGF, IL-6 and COX-2 proteins in granolusa cell layers was Semi-quantitative by Image J software.

2.12. Statistical Analysis

All the values obtained from this study were expressed as the mean ± standard deviation (SD). The two-way analysis of variance (ANOVA) test was used to evaluate differences between the four groups.
3. Results

3.1. Ovarian and peritoneal vascular permeability and ovarian weight

The results of vascular permeability determined using the EB dye are presented below. The concentration of EB dye was significantly increased in the ovaries and peritoneal vascular of OHSS group compared to the control (P <0.001) (Fig. 1A and 1B). Evans-Blue dye vascular penetration in OHSS+HBV and OHSS+Met groups were lower than the OHSS group (P <0.001 and P <0.05) respectively (Fig. 1A). OHSS+HBV and OHSS+Met groups had a significant decrease in vascular penetration of EB dye compared to the OHSS group (P <0.001 and P <0.01) respectively (Fig. 1B). The average ovarian weight and diameter in the OHSS group were higher than the Control group (P <0.001 and P <0.01) (Fig. 1C and 1D). As can be seen in (Fig. 1C), ovarian weight in the OHSS+HBV and OHSS+Met groups indicated a statistically decrease compared to the OHSS group (P <0.001 and P <0.01) respectively. On the other hand, ovarian diameter in both OHSS+HBV and OHSS+Met groups were lower than the OHSS (P <0.01 and P <0.05) respectively (Fig. 1D).

3.2. Effect of treatments on the secretion of hormones

The E2, T, LH, P4, and FSH Serum levels in rats experiencing OHSS were significantly higher than the control group (P <0.001, P <0.05, P <0.01, P <0.05, and P <0.05) respectively. While, the comparison of groups OHSS + HBV and OHSS showed a significant decrease in the serum E2 (P <0.01) and P4 (P <0.05). Also, in the OHSS+Met group a significant decrease in E2 (P<0.001), T (P<0.05), LH (P <0.01), P4 (P <0.01), and FSH (P <0.05) was observed compared to the OHSS group (Table 1).

| Table 1 |

<p>| Effects of HBV and metformin treatment on sex hormones levels in OHSS rats. Values are expressed as mean ± SD. *P &lt; 0.05, **P &lt; 0.01, ***P &lt; 0.001. († P &lt;0.05, †† P &lt;0.01, ††† P &lt;0.001; showed values of the OHSS compared to the control group). OHSS, Ovarian hyper stimulation syndrome, HBV, Honey bee venom. E2, 17-β-Estradiol. T, Testosterone. LH, Lutein hormone. P4, Progesterone. FSH, Follicle stimulating hormone. |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>E2 (ng/ml)</th>
<th>T (ng/ml)</th>
<th>LH (pg/ml)</th>
<th>P4 (ng/ml)</th>
<th>FSH (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.27±0.01</td>
<td>0.73±0.03</td>
<td>2.36±0.15</td>
<td>66.03±1.7</td>
<td>1644.76±126.6</td>
</tr>
<tr>
<td>PBS</td>
<td>0.3±0.01</td>
<td>0.8±0.01</td>
<td>3.1±0.04</td>
<td>67.36±0.88</td>
<td>1593.24±13.05</td>
</tr>
<tr>
<td>HBV</td>
<td>0.36±0.04</td>
<td>0.8±0.03</td>
<td>2.81±0.1</td>
<td>65.69±1.07</td>
<td>1631.13±29.81</td>
</tr>
<tr>
<td>OHSS</td>
<td>††† 1.41±0.3</td>
<td>† 0.93±0.06</td>
<td>†† 4.12±0.76</td>
<td>† 72.83±2.3</td>
<td>† 1448.63±65.05</td>
</tr>
<tr>
<td>OHSS+HBV</td>
<td>** 0.89±0.01</td>
<td>0.87±0.02</td>
<td>3.96±0.09</td>
<td>* 65.33±0.41</td>
<td>1474.05±25.95</td>
</tr>
<tr>
<td>OHSS+Met</td>
<td>*** 0.26±0.01</td>
<td>* 0.7±0.01</td>
<td>** 2.89±0.01</td>
<td>** 53.95±0.2</td>
<td>* 1591.21±44.97</td>
</tr>
</tbody>
</table>

### 3.3. Lipid profile and serum insulin concentration and serum CRP and IL-6 levels

The OHSS group showed a significant increase in insulin secretion, HOMA-β (P <0.001), and a decrease in HOMA-IR (P <0.001) compared to the control group (Fig. 2A). The comparison of groups OHSS + HBV and OHSS showed a significant decrease in insulin, HOMA-IR and HOMA-β levels (P <0.01). This significant decrease was also observed in the comparison of the OHSS + Met with OHSS for insulin (P<0.05), HOMA-IR (P<0.01), and HOMA-β (P<0.01) respectively. (Fig. 2B) showed that the OHSS group had a significant increase in LDL (P <0.05) and decrease in Chol and HDL (P <0.05 and P <0.001) serum concentration level. Furthermore, comparing the OHSS + HBV group with the OHSS group, a significant increase in Chol and LDL (P <0.05 and P <0.01) respectively and a decrease in TG (P <0.01) was observed. These changes were associated with a significant increase in Chol and HDL (P <0.05 and P <0.01) respectively, and a decrease in TG (P <0.05) in the OHSS+Met group compared to the OHSS group.

### 3.4. MDA and LDH assay

Compared with the control group, levels of IL-6 in serum, was a significantly higher in the OHSS group and HBV group (P <0.001, and P <0.05) respectively. Also, the results showed a statistically significant decrease of IL-6 in the OHSS+HBV and OHSS+Met groups compared to the OHSS group (P <0.01 and P <0.05) Fig. 3A). (Fig. 2B) showed a statistically significant increase in CRP in the OHSS group compared to the control group (P <0.001). This increase can also be seen in the groups of OHSS+HBV and OHSS+Met compared to the OHSS group (P <0.001 and P <0.05). LDH activity determination was based on measuring the conversion of pyruvate to L-lactate and MDA concentration was calculated with tetraethoxy propane and absolute ethanol as external standards of MDA. The LDH and MDA level of the OHSS group increased significantly compared to the control group (P <0.001), suggesting that the lipid system had been corrupted (Fig. 3C and 3D). However, the LDH level of the OHSS+HBV, and OHSS+Met groups was lower than the OHSS group (P <0.01, and P <0.001) respectively (Fig. 3A). Also, the MDA level
of the OHSS+HBV and OHSS+Met groups was lower than the OHSS group (P <0.01 and P 0.05) respectively (Fig. 3B). The results indicated that the metformin and HBV protected the cell membrane to a certain extent, by reducing the invasion of reactive oxygen and thus inhibiting lipid peroxidation.

3.5. Treatments affect IL-6, COX-2, and VEGF expression in ovary

The results obtained from separate and identify proteins by western blotting can be seen in (Fig. 4A). In the OHSS + HBV and OHSS + Met groups, a decrease in expression of IL-6, COX-2, and VEGF was observed in comparison with OHSS based on the obtained band thickness. This decrease was higher in the OHSS+Met group than the OHSS+HBV group.

The expression levels of VEGF, IL-6 and COX-2 proteins in the OHSS group showed a statistically significant increase compared to the Control group (P <0.001). Compared with OHSS group, a significant decrease was observed in the expression of VEGF, IL-6 and COX-2 in OHSS + HBV and OHSS + Met groups (P <0.001) (Fig. 4B).

3.6. Immunohistochemistry and histopathological evaluation

The strong staining intensity of VEGF revealed in all groups without significant difference among them. However, IL-6 and COX-2 staining percentage was increased in induced-ovarian hyperstimulation rats. There was no significant difference in IL-6 and COX-2 staining percentage among groups receiving HBV and Met. (Fig. 5).

4. Discussion

Some cytokines and growth factors, especially VEGF, as effective factors in inflammation, induce COX-2. Inhibition of COX-2 by non-inflammatory anti-steroidal drugs reduces the expression level of the above-mentioned (Murphy and Fitzgerald 2001; Liu et al. 2011; Li et al. 2014). According to the exact timing of COX-2 expression in the ovary, it has been called the alarm of the mammalian ovulatory clock (Sirois et al. 2004). In this study, by investigating the COX-2 protein levels, we attempt to confirmed its role in the function of OHSS ovaries and the effect of HBV on OHSS rats, as an inhibitor of COX-2 expression via NF-KB signaling pathway.

In 2001, Tokoyama reported that COX-2 was observed in granulosa cells of secondary and growing follicles and it is not expressed in primary and mature follicles (Tokuyama et al. 2001). This means that COX-2 expression stops when follicles reach to graph phase and the re-construction of COX-2 starts after LH peak (Popovic-Todorovic, Racca, and Blockeel 2018).
In this study, the induction model of OHSS in rats was associated with increasing levels of serum LH, which was consistent with the study of O’Brien (O’Brien et al. 2013). Richards et al. reported that an increase in LH level causes continuous induction of COX-2 expression in the granulosa cells, cumulus oophorus, and follicular fluid in this group (Richards JS et al. 2002). On the other hand, Davies showed that prostaglandins affect the secretion of LH and sometimes FSH by changing the GnRH levels. If rats are treated with prostaglandin, E2, LH secretion increased (Davies G et al. 2002). Thus, in this study, the high LH level was likely maintained in rats because of increased expression of COX-2 and a further increase in PG level. Therefore, given the exact timing of COX-2 expression in the ovary and its mutual interdependence with LH levels, it can be stated that dysregulation occurs in the timing of COX-2 expression in OHSS (Elia et al. 2013; Diao et al. 2007; Setia, Vaish, and Sanyal 2012; Sirois et al. 2004; Sobolewski et al. 2010; Taghavi et al. 2014; Takahashi et al. 2006). It is possibly a result of changes in LH level and/or the number or activity rate of LH receptors. Finding a large number of corpus lutea, measuring the level of LH, and increase in the number of androgens showed that LH in the OHSS group was kept at a high level and in this case, was consistent with Sirois studies (Sirois et al. 2004).

High levels of LH, not only affect the amount of COX-2 but also play a role in changing the cytokines that are effective in ovarian function. Interleukin 6 which is essentially controlled by nuclear factor kappa beta, NF-κB, stimulates and regulates protein synthesis of the acute phase, activates hypothalamic–pituitary–ovarian axis, and impairs the signal transduction through changing activities of serine-threonine kinases (Son et al. 2007; Ji Hyun Park et al. 2014; Jung Hyun Park et al. 2010). Plante showed the first evidence for secreting IL-6 by the ovarian in primary ovarian tumors and ovarian cancer cell lines. It was reported that granulosa cells of many species, including rats, fox, rabbit, and human, had the active site of IL-6 production (Plante et al. 1994). Tamura showed that FSH increased the production of IL-6 and a membrane protein, LHR, in granulosa cells in a dose-dependent manner (K. Tamura, Kawaguchi, and Kogo 2001; Kazuhiro Tamura et al. 2000). In the present study, it was observed that in OHSS rats, the IL-6 level was increased in granulosa cells of pre-ovulatory follicles, which production of a large number of them have induced by gonadotropins, and its secretion depended on FSH and LH (Kazuhiro Tamura et al. 2000; K. Tamura, Kawaguchi, and Kogo 2001). According to these reports, in the this study, although the emergence of corpora lutea in the ovaries of patients with OHSS can produce high levels of progesterone and estradiol but increased IL-6 in granulosa cells, prevents the significant increase in these hormone levels (K. Tamura, Kawaguchi, and Kogo 2001; Kazuhiro Tamura et al. 2000). Therefore, in this study, HBV was used to reduce the levels of these cytokines and it is observed that HBV effectively decreased the amount of IL-6 in the ovaries with OHSS. This significant reduction, not only leads to removing the inhibitory effect of IL-6 on the production of progesterone and estradiol, but also reduces the number of corpora lutea and granulosa cells that produce these hormones. Therefore, as expected, significant changes were not observed in hormone levels in the venom- treated OHSS group, compared to the OHSS group.

There are strong reports about the decrease in the activity and the synthesis of other inflammatory mediators by HBV (Karimi et al. 2012; L. Karimzadeh et al. 2012; Latifeh Karimzadeh et al. 2013);
Adolapine, a component of venome, has anti-inflammatory activity due to its ability to inhibit synthesis system of PG by COX-2 (Son DJ et al. 2007).

The inhibitory effects of venom on PLA2 and COX-2 activities and decreased levels of NO, IL-6, IL-1, and ROS were evaluated and found that HBV reduces edema, chronic arthritis, and cytokine levels in cell line RAW264.7 and this effect is similar to the inhibitory effect of indomethacin (COX-2 specific inhibitor) (Latifeh Karimzadeh et al. 2013). The anti-inflammatory effects of venom compared with N-hexane and ethyl acetate on the production of COX-2 and inhibition of NOS activity and NO production and inflammatory cytokines such as TNF-α and IL-1β. They observed that venom has a much stronger effect on suppressing the activity and mRNA expression of COX-2, in a dose-dependent manner, with no toxic effects (Kitsou et al. 2014).

The mechanisms of action of HBV in inhibiting the COX-2 expression and modulating the inflammation and oxidative stress in this syndrome are inhibition of the NF-κB activity (J. Chen and Lariviere 2010; Karimi et al. 2012; Merlo et al. 2011; Son DJ et al. 2007; Son et al. 2007). In this study, based on the researchers in humans as well as in rodents, the changes in COX-2, VEGF, and IL-6 expression were evaluated by western blotting technique. It is likely that reduced expression of this enzyme, in OHSS ovaries, was caused by changes in the NF-κB signaling pathway applied by HBV. Specific inhibitors of COX-2, such as meloxicam, inhibited COX-2, and VEGF and reduced symptoms of the ovarian hyperstimulation syndrome in rats (Quintana et al. 2008).

Some researchers stated that free fatty acids increase in obesity are accounted for primary ligands for Toll-Like receptors and act as a link between inflammation and obesity regulatory systems and oxidative stress systems (Cipollone 2005; Cipollone and Fazia 2006). Oxidative stress-induced by Reactive oxygen species (ROS) of mononuclear cells (MNC) activated by glucose, generates or promotes OHSS as well as local inflammatory ovarian responses (González et al. 2006; Goldstein et al. 2005). In this study, malondialdehyde (MDA) as a marker of lipid peroxidation, which is highly soluble in serum, was one of the most important markers in this field, and total antioxidant capacity was measured as an estimation of potential combinations of different antioxidants in the body of OHSS rats and the negative relationship was observed between antioxidant level, and insulin level or insulin resistance (IR-HOMA).

So, according to the results, the authors believe that every factor (here HBV) which leads to decreased expression of COX-2 and consequent reduction in oxidative stress, can be more complementary investigated as an effective strategy for improving the metabolic symptoms of OHSS. Following the HBV therapy investigated in this study, the amount of CRP, insulin-resistant index, and oxidative stress indexes was reduced and these results associated with histological and molecular findings are clear evidence for the therapeutic effects of HBV in this syndrome. These findings, based on the reduction of CRP and metabolic symptoms in OHSS samples treated with metformin, are consistent with Palomba (Palomba et al. 2011).

5. Conclusion
The ovary is a vital endocrine organ involved in developing OHSS. Prevention of OHSS has been widely discussed on the subject of assisted pregnancy methods. Identification of high-risk patients for OHSS before beginning effective therapeutic procedures is essential for ART success. OHSS Patients are at high risk of inflammation, thus the preventive attention to this syndrome was evaluated in this study and it was shown that HBV associated with metformin (as a drug proved to improve fertility, insulin resistance, and modulating inflammatory condition) were effective and it can be used to ameliorate the symptoms of this syndrome in women who undergo the process of gonadotropins stimulation.

Declarations

Acknowledgment

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Authorship

M.N. and L.K. designed the study, did the laboratory data, analyzed and interpret of data, draft the article, revised it critically for important intellectual content and approved the version to be submitted.

Availability of data and materials

Data and materials are available upon written request to the corresponding author.

Declaration of conflicting interests

The authors have declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Disclosure of conflicts of interest

The authors declare that they have no conflict of interests.

Ethics approval and consent to participate

There are no “human subjects” in this study. All ethical considerations were taken into account following the Helsinki Convention and the observance of animal rights, and experiments were performed after the approval of the Ethics Committee of the University of Kharazmi (Ethical Code 61694110).

References


32. Quintana, Ramiro, Laura Kopcow, Guillermo Marconi, Edgardo Young, Carola Yovanovich, and Dante A. Paz. 2008. “Inhibition of Cyclooxygenase-2 (COX-2) by Meloxicam Decreases the Incidence of


**Figures**
Figure 1

Vascular permeability of 10 EB dye in Peritoneal (A), Ovaries (B) and also ovarian weight (C), and Ovarian diameter (D) in experimental rats. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. († P <0.05, † † P <0.01, † † † P <0.001; showed values of the OHSS compared to the control group). EB: Evans blue.
Figure 2

Effects of HBV, OHSS+HBV and OHSS+Met on Glucose, Insulin, HOMA-IR, and HOMA-β (A), Chol, LDL, HDL, and TG (B) in the OHSS rats. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. († P<0.05, †† P <0.01, ††† P <0.001; showed values of the OHSS compared to the control group. OHSS, Ovarian hyper stimulation syndrome, HBV, Honey bee venom. Chou, Cholesterol, LDL, Low-density lipid. TG, Triglyceride. HDL, High-density lipid. HOMA-IR homeostatic model assessment values for insulin resistance. HOMA-IS homeostatic model assessment values for insulin sensitivity.
Figure 3

Serum IL-6 (A), CRP (B), and also liver content of LDH (C), and MDA (D). Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. († P<0.05, †† P <0.01, ††† P <0.001; showed values of the OHSS group compared to the control group). IL-6, Interleukine-6. CRP, Reaction protein. LDH, Lactate dehydrogenase. MDA, Malondialdehyde.
Figure 4

The Effects of HBV on the IL-6, VEGF and COX-2 expression in the OHSS+HBV and OHSS+Met groups in western blotting.

A), Statistical analysis of Western blotting results (B) Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. († P<0.05, †† P<0.01, ††† P<0.001; showed values of the OHSS compared to the control group).
Figure 5

Hematoxylin & eosin stained histology, immunohistology and semi-quantitative analysis of data for local expressions of the VEGF, IL-6 and COX-2 proteins in granolusa cell layers. The luteinized follicles (a), follicular fluid (b), granulosa cell (c), and theca cells (d). Values are expressed as mean ± SD. ***P < 0.001. (††† P<0.001; showed values of the OHSS compared to the control group. PBS, Phosphate-buffered saline; HBV, Honey bee venom; OHSS, Ovarian hyperstimulation syndrome; Met, Metformin; VEGF, Vascular endothelial growth factor; IL-6, Interleukin 6; COX-2, Cyclooxygenase-2.