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Vitamin K_2 saves the memory of ovariectomized rats against inactive Osteocalcin

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Abstract:

Ovariectomy (OVX) is followed by release of uncarboxylated osteocalcin (ucOC) outside the bone. UcOC is not always that good which is a matter of controversy. Menaquinone-7(MK-7) is an isoform of vitamin K_2 with multiple effects and closely adherent to Osteocalcin. This study was designed to investigate the effect of vitamin K_2 on memory following OVX and the role of vitamin k_2 in saving it . 32 female adult fertile albino rats were subdivided into four equal groups each containing 8 rats (Control, K_2, K_2OVX, OVX). K_2 and K_2OVX rats received MK-7 at a dose of 35mg/kg/Bw five days / week. Memory was tested in all four groups. In addition, serum levels of uncarboxylated osteocalcin and sex hormones were measured. Hippocampal activity of SOD, MDA and Glutathione peroxidase were evaluated. Also histopathological examination of brain was performed. The memory was deteriorated significantly in OVX groups but with intake of vitamin K_2 there was non significant deterioration. Serum ucOC significant elevated in OVX rats but in K_2OVX rats the elevation was significant lower. MDA was significant higher while SOD and Glutathione peroxidase were significant lower in ovariectomy groups. However, vitamin K_2 minimized these changes. In conclusion, vitamin K2 can reduce brain oxidative stress, preserve memory after ovariectomy.

Keywords:

Osteocalcin, Ovariectomy, vitamin K_2, Memory, Antioxidant.

Introduction:

During bone formation osteocalcin (OC) is produced by osteoblasts ***[1, 2]*** then binds to the carboxyglutamic acid (Gla protien) residues which is aided by vitamin K_2 due to its high affinity for calcium. The γ carboxylation leads to absorption of calcium to into the bone matrix and mineralization of bone. So, in cases of reduction of bone mineralization, the osteocalcin is freed into the systemic circulation ***[3].*** Osteocalcin is known to have may effects in body regarding glucose and insulin secretion regulation and metabolic adaptation to exercise ***[4, 5].***Moreover, osteocalcin could improve male fertility and prevent cancer development ***[6]***. Normally, ucOC passes Blood Brain Barrier (BBB) and binds to different brain regions including hippocampus to regulate some brain functions***[7]***.However, its effect in menopause have not studied yet.

Vitamin K2 is classified according to the prenyl units number to short-chain menaquinone-4; MK-4) and long-chain (MK-7, MK-8, and MK-9 ***[8].*** The MK-7 form is more lipophilic than K1 and MK-4, with longer half-life and longer availability in the circulation for several days to be handled by extra hepatic tissue ***[9].*** The anti inflammatory role of MK-7 enable it to protect brain cells from neurodegenerative diseases ***[10, 11].*** The naphthoquinone ring specially is the key for this neuroprotection ***[12]*** with regulation of sulfotransferase activity and growth factor/tyrosine kinase receptor (Gas 6/Axl) in the brain ***[13].*** Additionally, vitamin K_2 is known to improve bone quality, which in turn reduces fracture risk above the age of 50 ***[14].***

Ovariecotmy is identified as the animal model for menopause and due to ovum depletion and sudden hormonal withdrawal many health problems occurs. Decrease bone quality with increase risk for fractures ***[15]*** and cardiovascular problems ***[16].*** The changes in brain cognitive functions are under study recently including impaired memory by various degrees ***[17, 18]*** **.** Although ucOC has been a target for treatment of various metabolic disorders ***[19]***, its targeting value in brain disorders is still uncertain. Moreover, The use of vitamin K_2 after OVX to preserve memory is still unclear.

Materials and Methods:

Animals

32 fertile healthy female albino rats (weight=150-200 gm) were obtained from the animal house from faculty of veterinary medicine of Zagazig University. Animals not fertile or less than 150 gm were excluded . Rats were housed in groups (n=4) in standard cages (n= 8) under 12:12 h light–dark cycle, kept at a comfortable temperature (20 to 24 ºC) with free access to food standard chow and tap water. Rats were acclimatized to the testing room environment in animal behavior laboratory of physiology department (for 1 week) ***[20].*** The experimental protocol was approved by physiology department and by The Institutional Animal Care and Use Committee Zagazig University **(ZU-IACUC/3/F/42/2019).**

Then the animals were randomly divided into main two groups (n=16) and each of them was subdivided into 2 subgroups (n=8):

Group(I) control: sham operated with sunflower oil intake by oral gavage once daily 5 days per week for 10 weeks (Sigma –Aldrich, U.S.A, MFCD00132403).

Group(II) K_2: sham operated with MK-7 intake 35mg/ kg/bw by oral gavage once daily 5 days per week for 10 weeks (Sigma –Aldrich, U.S.A,900074-1MG).

Group(III) K_2OVX:ovariectomizedwith MK-7 intake 35mg/ kg/bw by oral gavage once daily 5 days per week for 10 weeks (Sigma –Aldrich, U.S.A, 900074-1MG).

Group(IV) OVX: ovariectomized with sunflower oil intake by oral gavage once daily 5 days per week for 10 weeks (Sigma –Aldrich, U.S.A, MFCD00132403).

Ovariectomy

The animals were fasted overnight, they were anaesthetized with 2% sodium pentobarbital by intra peritoneal injection (0.2ml/100g) ***[21].*** A central midline incision 0.5 cm was made with scalpel. The ovaries were palpated and removed bilaterally. In sham operated groups, the same procedures were done except that the ovaries were just palpated and not removed ***[22].*** Finally, there was any mortality.

Modified Barnes Maze test

A test for working memory and learning in rats. The maze consists of woody white circular platform elevated about 90 cm by aluminum support frame from the ground (diameter = 122 cm in diameter and thickness = 1 cm) with 20 equally spaced holes (diameter = 10 cm) around the periphery and only one of them was connected to the escape tunnel. A black woody start chamber was placed in the centre of maze (an opaque, 20 cm× 30 cm long, and 15 cm high, open-ended chamber). A removable black escape box 38.7 cm long × 12.1 cm wide× 14.2 cm in depth was placed under one hole of platform. 24 hours before the acquisition trial (day 0) habituation was done for all rats. On days 1-3 six acquisition trials were done in a form of 2 trials per day each of them was 3 minutes or until the rat enter escape box. On days 4 and 9 two probe trials were done each one was 2 minutes but without attached escape box. For each trial the latency to reach escape box and number of errors to reach escape box were calculated ***[23]***.

Blood samples and hippocampus Dissection

Rats were initially anesthetized with ether and blood samples were collected from retro-orbital sinus then s were centrifuged at 6000 × gm for 10 min and the serum was stored at -80 C ***[24]***. Then rats were sacrificed and the brain was removed from the skull then rinsed in ice cold saline to remove any surface blood. Then was placed on cold metal plate and cut bi-half into right and left hemispheres. The olfactory bulb was cut then frontal cortex then the ventral side of the brain was put up and the midbrain was removed to expose the hippocampus. then the hippocampus was removed. Finally, the hippocampal specimen was frozen in liquid nitrogen and stored at -80°C ***[25]***.

Tissue preparation

Hippocampal samples were homogenized with ice-cold phosphate buffered saline (PBS, pH 7.4) (100 mg tissue per 1 mL PBS). Then the resulted suspensions were centrifuged at 4 C with 4,000–6,000 RPM for 20 min then the supernatant was collected. The protein concentration of the SOD, MDA and Glutathione were measured by colorimetric technique ***[24].***

ELISA hormonal assay

Rat estradiol kits (E2): (BC-1111, Bio Check, Inc. 323 Vintage Park Dr. Foster City, CA 94404), Rat progesterone kits: (BC-1113, Bio Check, Inc. 323 Vintage Park Dr. Foster City, CA 94404), Rat lutinizing hormone kits (LH):(MBS2514287, BioSourcehOST-EASIA Kit, BioSource Europe S.A. Rue de I'Industrie, 8, B-1400 Nivelles, Belgium) and Rat Follicular Stimulating Hormone kits (FSH): (MBS2507988, Bio Sourceh OST-EASIA Kit, Bio Source Europe S.A. Rue de I'Industrie, 8, B-1400 Nivelles, Belgium) ***[26, 27].***

Serum uncarboxlated Osteocalcin (UOC)

ELISA kits, Catalog number: MBS2020904,BioSourcehOST-EASIA Kit, BioSource Europe S.A. Rue de I'Industrie, 8, B-1400 Nivelles, Belgium) ***[28].***

Hippocampal oxidative markers colorimetric assay

Rat malondialdehyde (MDA) kit ***[29]***, Rat superoxide dismutase (SOD) kit ***[30]*** and Rat glutathione peroxidase kit ***[31]***: (Egyptian Company for Biotechnology (SAE), Obour city, Cairo, Egypt.).

Histopathological study

Brain tissues were placed in formalin 10% and prepared for haematoxylin and eosin staining ***[32].***

Data analysis

SPSS 19 software (Inc. Chicago, IL, USA) was used for data analysis. The data is presented in Mean ± SD and the difference was assessed by one way ANOVA test with significance at $P<0.05$. Also person's correlation was calculated between OC and oxidative markers, r value was evaluated and the correlation was significant when P value <0.05

Results

Modified Barnes Maze test

Table (1): Latency to escape box in all the studied groups in seconds (mean ± SD).

Trial	AT1	AT2	AT3	AT4	AT5	AT6	PT1	PT2
Control	**48±4.40**	**30±5.07**	**38.88±2.85**	**30.25±2.25**	**26.76±3.11**	**19±1.51**	**17.25±2.12**	**12.75±3.06**
K_2	**48.75±5.42**	**31.36±6.44**	**38.25±3.56**	**31.25±4.68**	**25.5 ± 3.46**	**19.13±2.03**	**17.25±2.12**	**13.75±2.81**
K_2OVX	**67.88±8.59**	**31.38±7.11**	**35 ± 5.18**	**31.25±3.45**	**25.63±3.02**	**19.12±3.44**	**20.63±3.66**	**17.13±3.18**
OVX	**83.38±19.74**	**38.75±6.73**	**48.25±12.98**	**35.63±3.38**	**32.13±8.29**	**25.25±3.69**	**42.25±2.19**	**58.5 ± 4.11**

There was any significant difference between K_2 and control group (P value > 0.05). However, there was a significant longer duration acquisition trial 1 only in K_2OVX compared with sham operated groups (P value < 0.05). In OVX group there was a significant longer duration in probe trial 1 with P value <0.05 comparing to K_2OVX and P value <0.01 for sham operated groups while P value was <0.01 with all groups in all the remaining trials.

Table (2): Number of errors to reach escape box in all the studied groups (mean ± SD) .

Trial	**AT1**	**AT2**	**AT3**	**AT4**	**AT5**	**AT6**	**PT1**	**PT2**
Control	**4.5± 0.76**	**2.36 ±0.74**	**3 ± 0.93**	**1.38 ±0.52**	**2.36 ±0.52**	**1.5 ± 0.53**	**1.5 ± 0.53**	**0.63±0.52**
K_2	**4.63 ± 0.74**	**2.63 ±1.06**	**3 ± 0.76**	**1.5 ± 0.53**	**2.36 ±0.52**	**1.36 ±0.52**	**1.5 ± 0.76**	**0.88±0.59**
K_2OVX	**6.25 ±1.28**	**2.88 ±0.83**	**3.12±1.25**	**1.63± 0.52**	**2.5 ± 0.53**	**1.5 ± 0.76**	**1.5 ±0.53**	**1 ±0.53**
OVX	**7 ± 1.07**	**6.5 ±0.53**	**4.25± 0.89**	**3± 0.76**	**4.25 ±1.04**	**2.88 ±0.83**	**6.88 ± 0.99**	**5.5 ± 1.6**

There was any significant difference between K_2 and control group (P value > 0.05). However, there was a higher number of errors acquisition trial 1 only in K_2OVX compared with sham operated groups (P value < 0.05). In OVX group there was a significant higher number of errors in probe trial 1 with P value <0.05 comparing to K_2OVX and P value <0.01 for sham operated groups while P value was <0.01 with all groups in all the remaining trials.

Hormones

The ovariectomized groups had significant lower serum level of E_2 (K_2OVX= 199.75 ± 25.89 and OVX=190.63± 15.68) and progesterone (K_2OVX= 0.33 ± 0.11 and OVX= 0.34 ± 0.05) than the sham operated groups (Control= 345.25 ± 31.07, 5.88 ± 0.26 ± 0.26 and K_2= 341.88 ± 42.65, 6.02 ± 0.52 for E_2 and progesterone respectively) with P value <0.05. On the other hand, The ovariectomized groups had significant higher serum level of FSH (K_2OVX= 6.77 ± 1.45 and OVX= 6.7 ± 1.39) and LH (K_2OVX= 41.02 ± 5.23 and OVX= 43.05 ± 4.19) than the sham operated groups (Control= 1.97 ± 0.5, 10.13 ± 2.43and K_2= 2.01 ± 0.37, 9.86 ± 3.57 for FSH and LH respectively) with P value <0.05.

Uncarboxylated Osteocalcin (ucOC)

In sham operated groups serum levels of UOC were found to be 10.09 ± 0.92 Pg/ml in control and 8.97 ± 1.61 Pg/ml in K_2 group with no significant difference between them P value >0.05. However in K_2OVX was 13.12 ± 1.08 Pg/ml which is significantly higher than the sham operated groups P value <0.01. In the OVX group serum level of UOC was 14.67 ± 1.39 Pg/ml and this is significant higher than sham operated with P value <0.01 and K_2OVX with p value <0.05.

Oxidative markers

Regarding SOD activity in hippocampus in sham operated groups it was 3.66 ±0.07 and 3.63±0.07 u/gm for control and K_2 groups respectively with no significant difference (P value >0.05) . However, in K_2OVX the activity was 3.01± 0.49 u/gm which is significant lower than sham operated groups (P value <0.0). The activity of SOD was the most significant lower in OVX group comparing to other groups (1.16 ± 0.17 u/gm) with P value <0.01.

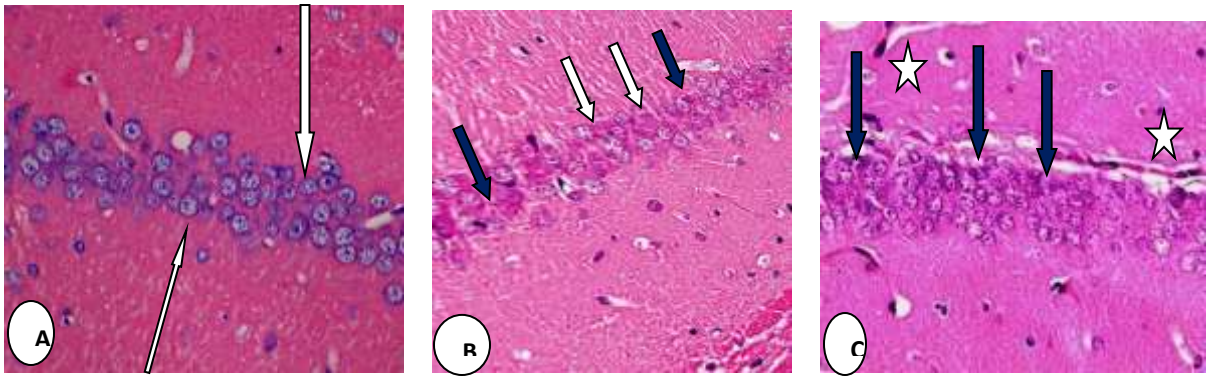
Similarly, Glutathione peroxidase activity in sham operated groups was 31.6 ± 3.96 and 31.4 ± 3.7 nmol/ gm for control and K_2 groups respectively without significant difference between them (P value > 0.05) . In the K_2OVX group the activity was significantly lower (22.36 ± 2.53 nmol/gm) with P value < 0.01. However, in OVX group the activity was significant lower than all groups (7.76 ± 1.4 nmol/gm) with P value <0.01.

On the other side, MDA activity was 9.19 ± 0.59 and 9.16 ± 0.47 nmol/ gm for control and K_2 groups respectively with no significant difference in between (P value >0.05). While the activity in K_2OVX was significantly higher 10.87 ± 1.25 nmol/ gm with P value >0.01. In the OVX group the activity showed significant increase comparing to other groups (12.8 ± 2.8 nmol/ gm) with P value <0.01.

Person's correlation

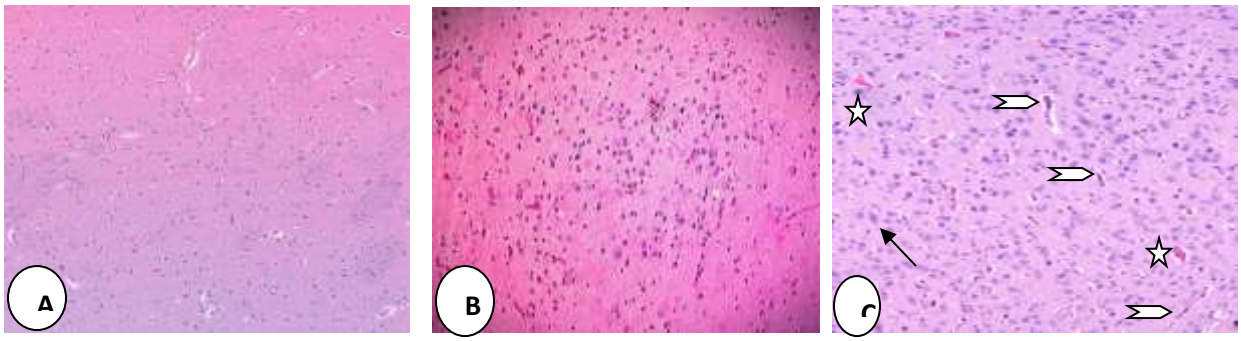
There was a negative correlation between serum ucOC and hippocampal SOD (r= -0.629 and r = -0.770 K2OVX and OVX respectively fig.5,6.) and Glutathione (r= -0.782 and r= -0.786 for K2OVX and OVX respectively fig.7,8.) only in the K2OVX and OVX groups. However, MDA was positively correlated with OC in the same groups (r= 0.636 and r= 0.727 for K2OVX and OVX respectively fig.9,10) and for all parameters P value was <0.05.

Histopathology



Photomicrograph (1): Haematoxlyin and Eosin staining of section of CA3 region of hippocampus at magnification of 400M .

A) Control group: Shows normal cells with multiple nuclei (Arrow) and no signs of necrosis or engorged blood vessels.
B) K2OVX group: Shows normal cells (white arrow) , very few dark neurons as a sign of degeneration (dark arrow).
C) OVX group: Shows multiple dark neurons (dark arrows) as a sign of degeneration with engorged blood vessels (star).

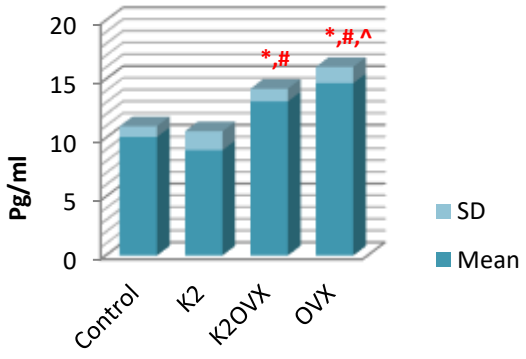


Photomicrograph (2): Haematoxlyin and Eosin staining of section of brain tissue at magnification of 200M .

Control group: Shows normal brain parenchyma with normal neurons . no signs of neuronal loss , degeneration or vessels engorment.

K2OVX group: Shows normal brain tissue which is not significant different from the control group.

OVX group: Shows great neuronal degeneration (black arrows) , vascular enngorment (stars) and mineralization of some microblood vessels (arrow heads).



figure(1): Shows serum level of uncarboxylated Osteocalcin in all the studied groups.

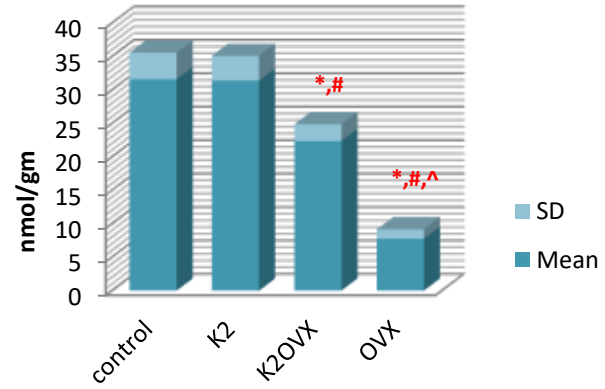
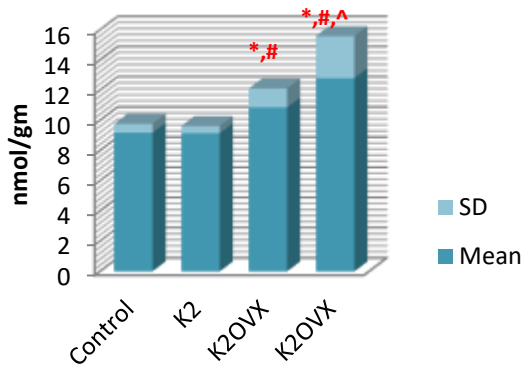
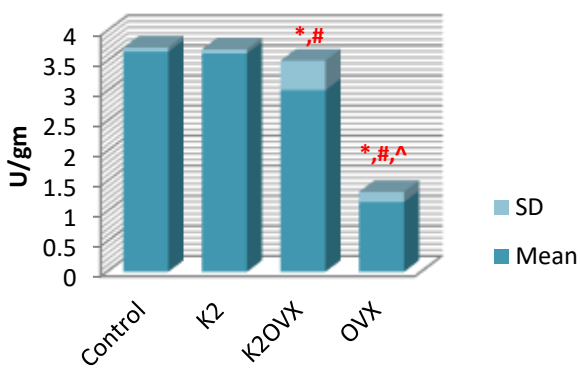


figure (2): Shows glutathione levels in hippocampus in all the studied groups.



figure(3): Shows MDA levels in hippocampus in all studied groups.



figure(4): Shows SOD levels in hippocampus in all the studied groups.

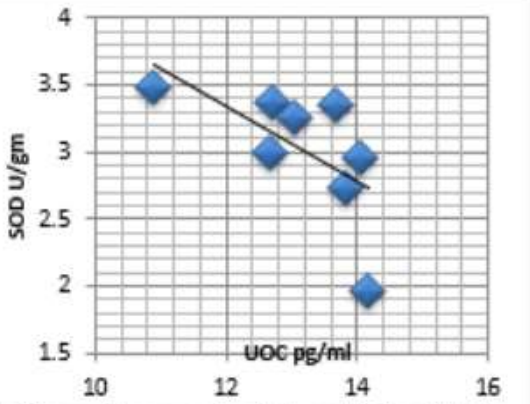


fig (5): negative correlation between UOC and SOD in K2OVX group.

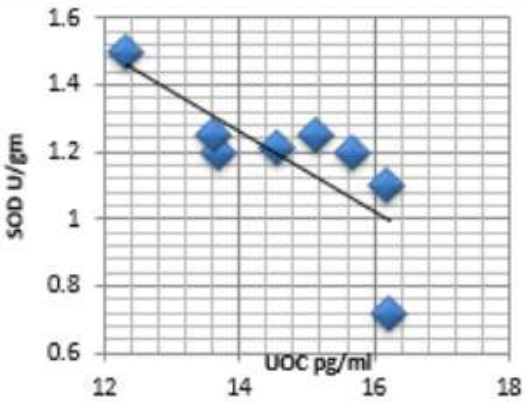
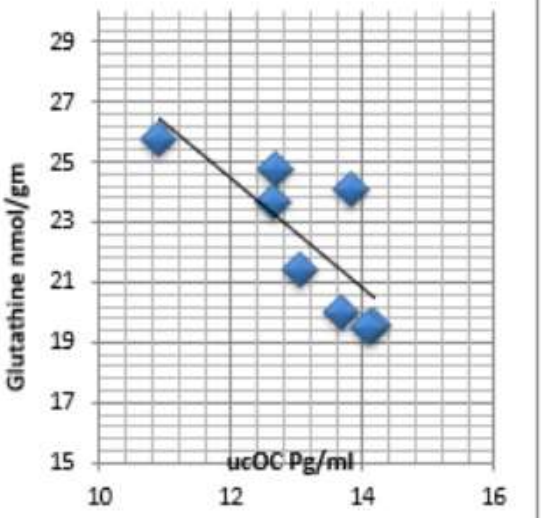
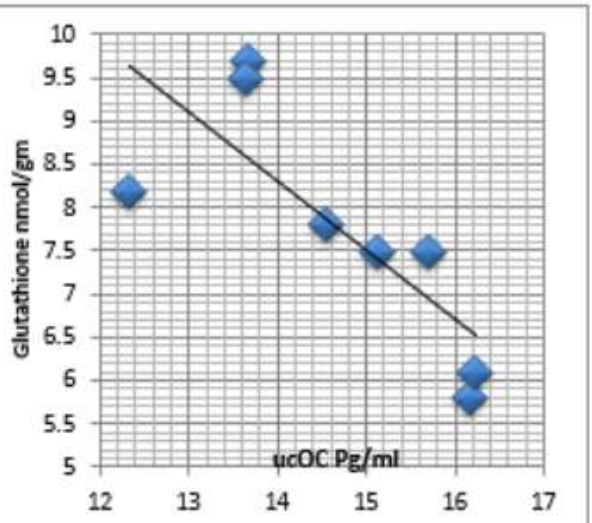


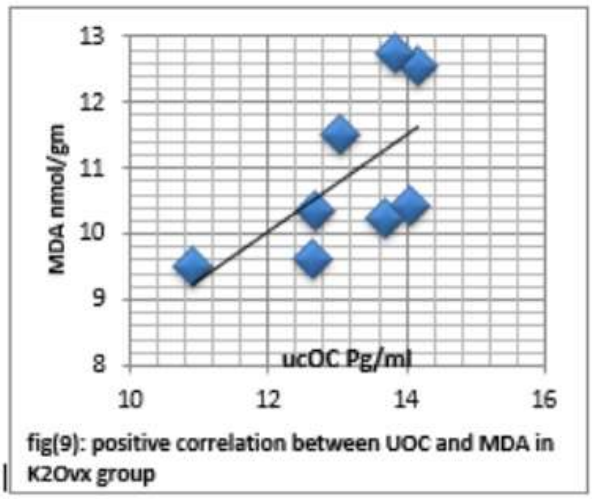
fig (6): negative correlation between UOC and SOD in OVX group.



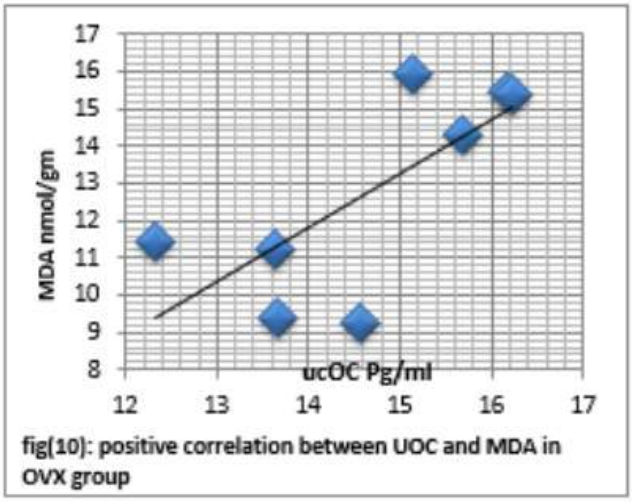
fig(7): negative correlation between UOC and Glutathione in K2OVX group.



fig(8): negative correlation between UOC and Glutathione in OVX group.



fig(9): positive correlation between UOC and MDA in K2Ovx group



fig(10): positive correlation between UOC and MDA in OVX group

Discussion

Menopause always associated with impairment of many physiological processes in women particularly brain cognitive functions due to deficiency of sex hormones ***[33].*** The present study showed a significant decline in E2 and progesterone serum levels in ovariectomy groups comparing to the sham operated groups in accordance of the well known endocrinal profile of ovariectomy ***[34].***

OC is a bone matrix γ-carboxyglutamate (Gla-protien) and its normally trapped in the bone in carboxylated form. Once demineralization occurs as in OVX it released as ucOC in the serum ***[32, 35]***.MK-7 is effective in gamma carboxylation of OC than other forms of vitamin K_2 and even in low doses can reduce the serum level of ucOC ***[36].***Peripherally, OC can regulate the osteoblastic function , insulin, adiponectin secretion, body fat mass, serum triglycerides levels and male fertility. While central, OC mainly in the uncarboxylated form binds to thalamus , hypothalamus, brainstem and hippocampus for synthesis and regulation of many chemical transmitters ***[7, 37].***

In this experiment, serum level of ucOC was significantly higher following the overiectomy. However, the administration of MK-7 in the OVX lead to significant decrease in its level although it was still significantly higher than the control groups. In this line, previous studies demonstrated that serum level of ucOC in early menopause is increased due to high bone turn over and decreased by about 28% with MK-7 alone or combined with other drugs ***[28, 38].*** While other studies found no difference in serum OC level before and after menopause ***[39, 40].***Moreover, ***Al-Daghri et al., 2015*** found that serum OC decreased with menopause and no other studies support their findings ***[41].***

Modified Barnes Maze test was used to assess short , long term memory and learning. The OVX group spent a significant time in reaching the escape box and had a significant number of errors until reaching the escape box through all the acquisition trials and probe trials 1 and 2 comparing to the other groups. These findings indicate disturbed memory and learning ***[23]*** in OVX rats not receiving MK-7 but this disturbances have been reversed by receiving MK-7 intake. Similary, more studies showed impairment of short, long term and spatial memory after

ovariectomy as estrogen is necessary for normal hippocampal functions***[42, 43]***. Moreover, ***[44]*** proved that short term memory disturbance is common finding following OVX and associated with mood disorders. ***[45, 46].*** Controversely, previous study didn't prove any effect of MK-7 on the memory of rats ***[47]***.

The increased of LH level occurring in menopause leads to increase plasma level of Amyloid $_{B1-40}$ and Amyloid$_{B1-42}$ that leads to impaired memory function ***[48].*** In addition, the interaction between ERs and NMDA receptors and its action on phosphatidyl inositol 3-kinase (PI3 K) and extracellular signal-regulated kinase (ERK) are affected ***[49, 50].*** Following OVX, the estrogen depletion leads to production of free oxygen radicals with reduction of antioxidant enzymes, SOD and GPx with increased MDA and disturbed SOD/ Peroxidases ratio in hippocampus ***[51]***. Moreover, there is increase in inflammatory cytokines in hippocampus with decrease anti-inflammatory cytokine that results in change microglial polarization ***[52].***

In this line, hippoccampal oxidative markers were measured in our study (SOD, MDA, GPx) and the level of SOD and GPx were significantly lower in the OVX group but MDA was significantly higher. SOD and GPx showed significant negative correlation with ucOC serum level in ovariectomy groups only but the MDA level showed a significant positive correlation. However, the intake of MK-7 in OVX rats lead to a significant improvement in these parameters. Which is a good indicator for the role of MK-7 in increasing the anti-oxidative markers.

MK-7 and OC have anti-inflammatory action and can reduce the productions of pro inflammatory genes ***[53, 54].*** Hippocampus as a centre for many cognitive function is characterized by disturbed glucose metabolism after OVX and OC could maintain glucose metabolism in brain through CLUT 1 , 4 ***[7, 55].*** In accordance with our results, MK-7 supplementation protected the astrocytes from the hypoxic damage and reduce the ROS levels ***[56].***

Although the uncarboxylated form of osteocalcin is the most suitable for passing blood brain barrier (BBB) and performing its central action ***[7]***, in our study the increased ucOC level was associated with worsening of memory and oxidative profile. Depending on a previous study on aorta, the high level of ucOC in serum as inactive matrix Glap-protien resulted in vasoconstriction and calcification in aortic wall ***[57],*** the histopathological examination of brain tissue was done. The result showed signs in neuronal damage in hippocampus of OVX group with capillary engorment however in the K_2OVX group the damage was rare and with no significance when compared to control. In addition, the examination of the remaining brain tissue revealed marked neuronal damage and capillary engorment with calcification in wall of some micro vessels only in OVX group. We could explain that by elevated ucOC resulted in microvascular calcification with subsequent oxidative damage and neuronal degeneration and the positive correlation proved in this study between ucOc serum level and oxidative markers favors this. With MK-7 intake, the carboxylation of osteocalcin protect the brain from this undesired effect.

Conclusion

Vitamin K_2 can be used as a prophylaxis against memory deterioration occurring after OVX. Its direct anti-oxidative effect or indirect through gamma carboxylation of osteocalcin to prevent brain oxidative damage are the underlying mechanisms. UcOC not always good for the brain specially after OVX. Further studies are needed to investigate other possible mechanisms

connecting vitamin K2 and OC at the brain level. Also the effect of high ucOC levels on other brain functions needs further investigations.

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Competing interests statement

The authors declare no competing interest.

Data availability

All data are available from the corresponding author upon request.

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