

Preventing Effect of Vitamin E on Oocytes Apoptosis in Morphine-treated Mice

Efecto Preventivo de la Vitamina E sobre la Apoptosis de Ovocitos en Ratones Tratados con Morfina

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SUMMARY: Several studies have shown that Morphine Sulfate affects on fertility, embryogenesis and consequent pregnancy loss and ultrastructural alterations of oocytes in animal model. This study was done to determine the effect of morphine sulfate on oocytes apoptosis and preventive role of daily supplementation of Vitamin E on oocytes apoptosis in morphine sulfate -treated mice. Twenty-four NMARI female mice were randomly allocated into four experimental groups. For 15 days, control group received saline (0.2 ml/day by subcutaneous injection), group I Vitamin E (60 mg/kg/day orally), group II Morphine Sulfate (10 mg/kg/day by subcutaneous injection) and group III Morphine Sulfate with Vitamin E (60 mg/kg/day orally). Then, animals were superovulated with PMSG (10 Units) and 10 Units of HCG. The next day the animals were sacrificed, oocytes were flushed from each fallopian tube. The collected oocytes were subjected to determine apoptosis by Tunnel assay with using Fluorescent Microscope. According to our results, the number of retrieved oocytes were 121, 132, 86 and 114 in control, experimental group I, II and III, respectively. Morphine Sulfate treatment increased apoptosis in oocytes to 17.44% whereas oocytes apoptosis was 4.13% in Controls. Supplementation with Vitamin E in Morphine Sulfate -treated mice reduced the oocytes apoptosis to 7.01%. This study showed that Morphine can increase apoptosis in oocytes and Vitamin E treatment significantly reduces oocytes apoptosis in the Morphine Sulfate -treated mice.

KEY WORDS: Oocytes; Morphine sulfate; Vitamin E; Apoptosis; TUNEL assay; Mice.

INTRODUCTION

Opium substance consumption in young people is increased in comparison with last decade. More than 90 percent of women consuming Morphine Sulfate as a component of opium takes place in childbearing age (Vucinovic *et al.*, 2008; Dehghan *et al.*, 2010).

Opium addiction causes menstrual period, reduces CNS repose to excitation of visceral nerve in cervix of uterus, decrease of oxytocin secretion and myometrium contraction (Shin & Eisenach, 2003; Kayacan *et al.*, 2007; Yoo *et al.*, 2001; Nacitarhan *et al.*, 2007; Kowalski *et al.*, 1998).

Morphine Sulfate (C17 H19 O3 N) is one of the 40 alkaloids present in opium from *Papaver somniferum* which as one of the main opium substances causes disorder in uterus

cycle, reduce of pregnancy chance and inhibition of normal of ovulation in mice (Siddiqui *et al.*, 1997; Lakhman *et al.*, 1989). Morphine Sulfate reduces volume of placental blood, length and weight of fetuses and cerebral cortical layers and number of neurons in frontal lobes (Sadraie *et al.*, 2008). Morphine Sulfate can transfer from placenta barrier and due to morphine receptor in placental vile and fetal tissue has long time effects (Kopecky *et al.*, 1999; Ray & Wadhwa, 1999).

Opioids have oxidative properties and due to this spatiality increase apoptosis in many of cells by producing free radicals (Singhal *et al.*, 1994; Pan *et al.*, 2005). Several studies have shown that Morphine Sulfate increase apoptosis in neurons, glial, hepatocytes, cells of immune systems and epithelial cells in endometrium of uterus (Dehghan *et al.*;

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Lin *et al.*, 2009; Li *et al.*, 2009; Wang & Han, 2009; Svensson *et al.*, 2008; Singhal *et al.*, 1999; Payabvash *et al.*, 2006).

Morphine sulfate can increase the generation of free radicals by acting as a pro-oxidant (Singhal *et al.*, 1994).

Lipid peroxidation increase following blocking of anti-oxidant enzyme. This process leads to the formation of free radicals or reactive oxygen species (ROS) (Niki *et al.*, 2005). These free radicals or ROS caused damage to the cell membrane and DNA fragmentation (Yang *et al.*, 1998). Also, Oxidative stress cause chromosomal instability and program cell death (Kumar & Cotran 2003). Tunnel assay is a well develop method for evaluation of apoptosis or program cell death in cells (Gavrieli *et al.*, 1992; Paszkowski *et al.*, 2002). Program cell death is known as main mechanism in oocytes death (Morita & Tilly, 1999; Tilly, 2001). Also studies have shown that anti-oxidant component reduce the oxidative stress in liver tissue (Zhang *et al.*, 2004).

Vitamin E as a lipid-soluble substance has anti oxidant properties (Brigelius-Flohe & Traber, 1999). Vitamin E has important role in prevention of lipid production and oxidative stress reaction by scavenging free radicals. Due to anti oxidant properties (Brigelius-Flohé & Traber, 1999; Sheppard *et al.*, 1993; Burton & Ingold, 1986), It can reduce the senile oxidative stress reaction on number and quality of oocytes (Tarín *et al.*, 2002).

Furthermore, several studies have shown that Vitamin E and its various components reduce the oxidative stress reaction by scavenging free radicals in cells and cell organelles (Hassa *et al.*, 2007; Kunitomo *et al.*, 2009; Sen *et al.*, 2007; Mokhtar *et al.*, 2009). With Regard to impotent the number of oocytes in fertility and increase of opium consumption in child- bearing age women, this study was done to determine the effect of daily supplementation of Vitamin E on oocytes apoptosis in morphine sulfate -treated mice.

MATERIAL AND METHOD

Female NMARI mice weighing 20-25 g and aged 8-10 wk were obtained from the pastor institute and all procedures were performed with approval from the animal ethics committee of The Golestan University of medical sciences in Iran. The animals were maintained in a climate-controlled room under a 12-hour alternating light/dark cycle (09:00–21:00 hours in light), 20.1°C to 21.2°C temperature, and 50% to 55.5% relative humidity. Dry food pellets and water were provided *ad libitum*.

Twenty-four female mice were randomly allocated to four separate groups of six animals each control group (Group C) received saline (0.2 ml/day by subcutaneous injection). Animals in Vitamin E treated group (I) were received Vitamin E (T-3251; Sigma) (60 mg/kg/day orally). Animals in Morphine Sulfate -treated group (group II) were received Morphine Sulfate (10 mg/kg/day by subcutaneous injection). Also Female mice in Morphine with Vitamin E treated group (group III) were received Morphine Sulfate (10 mg/kg/day by subcutaneous injection) with Vitamin E (60 mg/kg/day orally). The treatments were scheduled for 15 consecutive days.

Superovulation protocol. The animals were superovulated following these schedules. Mice were treated with an intraperitoneal injection of 10 IU pregnant mare serum Gonadotrophin (PMSG; Intervet International B.V., Netherlands). Human chorionic gonadotropin (10 IU; Intervet International B.V., Netherlands) was injected intraperitoneally 48 hours later. The next day the animals were sacrificed by cervical dislocation, oocytes were flushed from each fallopian tube using a hypodermic needle with media (a Mem) under a dissecting microscope, and the retrieved cumulus oocytes complexes were examined by TUNEL Assay.

TUNEL method. The retrieved cumulus oocyte complexes were incubated for 60 minutes in 2% paraformaldehyd and 2 minutes in triton X solution. Tunnel assay (*In situ* cell death detection kit- Roche Germany) was used to determine Failure rate of DNA (DNA damage). Incubation with Propidium Iodide (P4170; Sigma) solution was used for background staining (Hassa *et al.*; Kunitomo *et al.*). Then Failure rate of DNA were determined by florescent microscope (BX-51 Nikon –Japan) with Excitation wave length in the range of 450-500nm and detection in rang of 515-565 nm.

Statistical analysis. Data analyzed by One way ANOVA and Post Hock tests. A P value less than 0.05 was taken as statistically significant.

RESULTS

The number and percent of retrieved and apoptotic oocytes of mice in four experimental are depicted in Figure 1.

According to our results, the number of retrieved oocytes were 121, 132, 86 and 114 in control, experimental group I, II and III, respectively.

The number and percent of apoptotic oocytes was 5 (4.13%), 7 (5.30%), 15 (17.44%) and 8 (7.01%) in control,

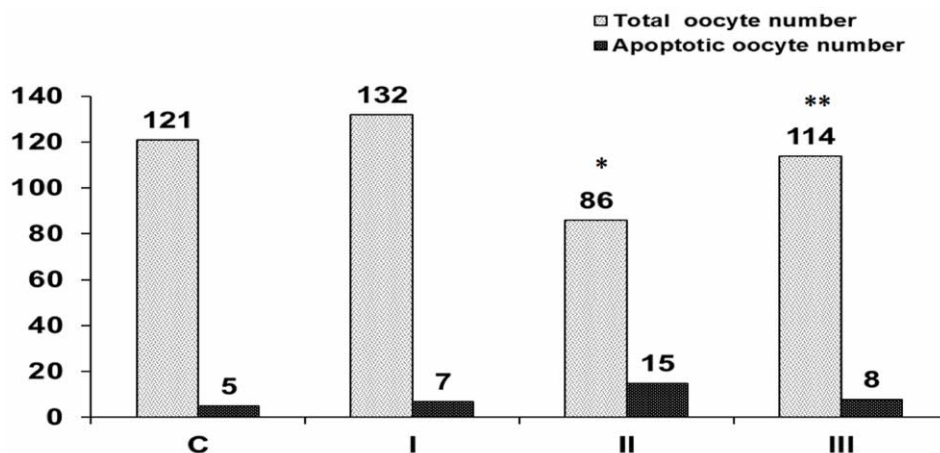


Fig. 1. The number oocytes and apoptotic oocytes in control (C), vitamin E (I), Morphine (II) and Morphine and Vitamin E (III) groups.

*P<0.05 In morphine treated (II) in comparison with control(C) and morphine with Vitamin E (III) groups.

**P>0.05 in morphine with Vitamin E (III) group in comparison with control(C) and Vitamin E (I) groups.

Vitamin E treated mice, Morphine Sulfate treated and nicotine and Vitamin E treated mice, respectively.

Oocyte apoptosis significantly increased in Morphine Sulfate treated mice in comparison with controls ($p < 0.5$). Also oocytes apoptosis in Morphine Sulfate –vitamin E group significantly reduced in comparison with Morphine Sulfate treated mice ($p < 0.5$). There was no significant difference between apoptotic cell number in controls and Morphine Sulfate –vitamin E group.

DISCUSSION

This study showed that Morphine Sulfate exposure increased apoptosis in mice oocytes. Our findings are comparable to previous studies (Dehghan *et al.*; Pan *et al.*; Singhal *et al.*, 1999; Zhang *et al.*; Nasiraei-Moghadam *et al.*, 2010; Bhat *et al.*, 2004).

Degahn *et al.* reported that interaperitoneal administration of morphine 10 mg/kg for 15 days in mice dams cause signs of apoptosis and inflammation with increase of blood vessels in uterus. Furthermore disruptions of nuclear envelop in epithelial cells of endometrium and irregular space between nucleolus and chromatin was founded.

Also, Singhal *et al.* (1999) have indicated that morphine with increase of free radicals such as SOD (super oxide desmotase) and katalase cause increase of gene expression of BAX and Caspase 3 and reduce of Bcl2 and increase apoptosis in T lymphocytes and Jurkat cells.

In other study, Nasiraei-Moghadam *et al.* reported that oral administration of morphine sulfate in mice dams during the first days of gestational age cause increase of gene expression of BAX and reduce of Bcl2 and increase apoptosis in neuroblasts of offspring. Also, Pan *et al.* showed that heroin administration in mice reduces endogen antioxidants in blood stream and increase of ROS in white cells and finally it causes oxidative damage in proteins and lipids of brain and liver.

Morphine sulfate by production of NO (nitric oxide) and dysfunction of NADPH oxidase produce super oxide (O₃), this process cause binging of apoptosis in macrophages (Bhat *et al.*).

Furthermore, Zhang *et al.* reported that interaperitoneal administration of morphine sulfate cause DNA damage and increase of oxidative enzymes in hepotocytes. He also indicated that anti- oxidants such as ascorbic acid and Glutathione reduce negative effects of morphine in these cells. Thus, we can conclude that morphine sulfate increase Lipid peroxidation following blocking of anti-oxidant enzyme. This process leads to the formation of free radicals or ROS (Niki *et al.*). These free radicals or ROS caused damage to the cell membrane and DNA fragmentation (Yang *et al.*). Also, Oxidative stress cause chromosomal instability and program cell death (Kumar & Cotran). Program cell death is known as main mechanism in oocytes death (Morita & Tilly; Tilly).

Also, in our study Supplementation with Vitamin E reduced the oocyte apoptosis rate in morphine sulfate-treated mice. This reduces of oocyte apoptosis can be due to antioxidant properties of vitamin E.

Vitamin E and its various components reduce the oxidative stress reaction by scavenging free radicals in cells and cell organelles (Brigelius-Flohé & Traber; Sheppard et al.; Burton & Ingold).

Vitamin E, in fact, is the major peroxy radical scavenger in biological lipid phases such as membranes. Its antioxidant action has been ascribed to its ability to chemically act as a lipid-based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation and protecting the organism against oxidative damage (Hassa et al.; Kunitomo et al.; Sen et al.; Mokhtar et al.). Several studies reported that with vitamin E as an antioxidants substance has preventive effects (Zhang et al.; Hassa et al.; Train et al.).

Zhang et al. showed that anti-oxidants such as ascorbic acid and Glutathione reduce negative effects including DNA damage and increase of oxidative enzymes due to morphine in hepatocytes. Also, Hassa et al. in an experimental animal study reported that fertilization and cleavage rates were affected mainly in the smoke-exposed female mice population and treatment with vitamin E did

affect the fertilization, cleavage, and embryo development rates of smoke-exposed female mice. Indeed, Train et al. in an animal model have shown that oral administration of antioxidant (vitamin E and C) can counteract the negative effect of female aging on number and quality of oocytes.

CONCLUSION

This study showed that morphine sulfate exposure increases apoptosis in oocytes and Supplementation with Vitamin E reduce the oocytes apoptosis in morphine sulfate-treated mice.

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RESUMEN: Diversos estudios han demostrado que el sulfato de morfina afecta la fertilidad, embriogénesis y en consecuencia pérdida de la preñez y alteraciones ultraestructurales de los ovocitos en el modelo animal. Este estudio determinó el efecto del sulfato de morfina sobre la apoptosis de los ovocitos y papel preventivo de la suplementación diaria de la vitamina E en la apoptosis de ovocitos en ratones tratados con sulfato de morfina. Veinte y cuatro ratones NMARI hembras fueron asignados al azar en 4 grupos experimentales. Durante 15 días, el grupo control recibió solución salina (0,2 ml/día por inyección subcutánea), el grupo I vitamina E (60 mg/kg/día por vía oral), el grupo II Sulfato de morfina (10 mg/kg/día por inyección subcutánea) y el grupo de III sulfato de morfina con vitamina E (60 mg/kg/día por vía oral). Posteriormente, los animales superovularon con PMSG (10 unidades) y 10 unidades de HCG. El día siguiente, los animales fueron sacrificados, los ovocitos fueron aspirados desde cada tubo uterino. Los ovocitos recogidos fueron utilizados para determinar la apoptosis mediante el ensayo de TUNEL con el uso de microscopio de fluorescencia. El número de ovocitos recuperados fueron 121, 132, 86 y 114 en los grupos control y experimental I, II y III, respectivamente. El tratamiento con sulfato de morfina aumentó la apoptosis en los ovocitos un 17,44%, mientras que la apoptosis de los ovocitos fue 4,13% en los controles. La suplementación con vitamina E en los ratones tratados con sulfato de morfina redujo la apoptosis de los ovocitos en 7,01%. Este estudio demostró que la morfina puede aumentar la apoptosis en los ovocitos y el tratamiento vitamina E redujo significativamente la apoptosis en los ovocitos de ratones tratados con sulfato de morfina.

PALABRAS CLAVE: Embriones; Sulfato de morfina; Vitamina E; Apoptosis; Ensayo TUNEL; Ratones.

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