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Effects of antioxidant (*Salvia officinalis* L. extract) on *in vitro* fertilization and micromanipulation intracytoplasmic sperm injection in Albino mice

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Abstract. *The aim of this experimental study was to assess the effects of antioxidant (Salvia officinalis L. extract) on fertilized percentage of sperm. Ability and efficacy of treated sperms by two methods of fertilization (IVF and ICSI) in albino mice male. Ten males treated intra peritonially with S. officinalis L. for 35 days, sperm motility, sperm dead and abnormality were (86.43%±6.54, 17.50%±3.01, 13.39±2.04) respectively compared to control group (72.31%±3.92, 26.23%±2.94, 24.33%±2.91). Thirty adult mice females were treated with ECG and hCG to obtain super ovulation to increase numbers of oocytes. Females were sacrificed by dislocation of cervical vertebrate. Oviducts were removed and placed in a sterile disposable Petri dish containing 1ml Phosphate buffer solution (PBS). After collection of sperms from treated males, and follow method of in vitro fertilization and intracytoplasmic sperm injection, results of oocytes fertilized by IVF were 65.97%, while oocytes fertilizing by ICSI method were 61.53% compared to control sperms without treated by Salivia Officinalis (43% for IVF and 45.45% for ICSI).*

Keywords: *Salvia officinalis*, antioxidant, spermatogenesis, *in vitro* fertilization

Abbreviations: IVF – *in vitro* fertilization, ICSI – intracytoplasmic sperm injection, IUI – intrauterine insemination, LPO – lipid peroxidation

Introduction

Sage, *Salvia officinalis* L. (Lumiaceae) is an aromatic perennial plant native to southern Europe and Asia Minor. It is cultivated as a culinary herb and as a plant of great medicinal importance, officially listed in pharmacopoeias of many countries throughout the world. Leaf extracts of this plant have antibacterial (Farag et al., 2005), antiviral (Tada et al., 2000), anti-inflammatory (Baricevic et al., 2001), antihydrotic (Leung, 1999) and antioxidant properties (Miura et al., 2000). It has been shown that antioxidant properties of sage extracts have been attributed to their major abietane-type diterpenoids: carnosic acid (CA) and carnosol (Car) as well as rosmarinic acid (RA) (caffeoyl derivative) (Miura et al., 2000). Both carnosic acid and carnosol have been shown to be stronger antioxidants than synthetic antioxidants (butylated hydroxytoluene and butylated hydroxyanisole) in assays of their activity by the Rancimat method (Chen and Ski, 1992). These diterpenoids were also shown to inhibit superoxide anion production in the xanthone oxide system (Haraguchih, 1995).

The leaf extracts of *Salvia officinalis* have antioxidant activity exhibit strong antioxidant activity, largely attributable to various phenolic constituents including phenolic diterpenes such as carnosol and hydroxycinnamic acid derivatives, notably rosmarinic acid (Lamaison et al., 1991; Wang et al., 2000).

The IVF and ICSI techniques used to treated problem may be associated with low sperm production (oligospermia), poor sperm motility (asthenospermia), or abnormal morphology (teratospermia). Abnormal sperm function may also be evaluated with sperm function tests that evaluate sperm interaction with cervical mucus (cervical mucus penetration test), the zona pellucida surrounding the oocyte (hemi-zona binding assay), or the oocyte itself (hamster-egg penetration assay). Assisted reproductive techniques include intrauterine insemination (IUI), *in vitro* fertilization (IVF), and IVF with micromanipulation (Peter, 1997).

The demonstration of fertilization and live births by Palermo et al.(1996) was the first successful application of ICSI. Since that time, ICSI has been performed extensively in multiple centers to treat patients with severe male factor infertility. The success of ICSI procedures has been related to several factors (Farag et al., 2005) the viability of the spermatozoon, (Tada et al., 2000) the quality of the oocyte, (Baricevic et al., 2001) effective activation of the oocyte, and (Leung, 1999) ability of the oocyte to tolerate intracytoplasmic manipulation (Palermo et al., 1996).

Most clinical series report on using ICSI in cases where standard IVF is highly unlikely to succeed, that is, in patients with less than 500,000 motile sperm present in the ejaculate, or less than 4% normal forms with strict criteria evaluation (Liu and Baker, 1992). In addition, couples who have failed to fertilize any oocytes in a prior IVF cycle are considered appropriate candidates for IVF-ICSI. We have proposed the following indications for ICSI (Schlegel, 1997): a) sperm concentration < 2×10^6; b) sperm motility < 5 %; c) strict criteria normal morphology < 4 %;d) use of surgically retrieved spermatozoa; e) failure of fertilization in a previous IVF cycle.

Although fertilization and pregnancy rates with ICSI are similar or better than those achieved with normal sperm in other couples undergoing IVF. Oral antioxidant treatment appears to improve IVF and ICSI outcomes in those patients with sperm DNA damage, in whom this treatment reduces the percentage of damaged spermatozoa (Kodama et al., 1997).

In this study, the effect of *Salvia officinalis* on IVF, especially IVF with the advanced micromanipulation technique of ICSI, as tools for improved percentage of fertilization was investigated.

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Material and methods

Plant material Crude extracts (Aqueous extract)

Salvia officinalis were purchased from the local markets and identified in a Biotechnology Research Centre-Al-Nahrain University. The leaves were cleaned and finely powdered and extracted by: 50 g of plant powder was extracted with 250 ml of distilled water by reflex apparatus for 2 hour at (60-90) degree centigrade, then the water was removed under reduced pressure by rotary evaporator at 60ºC, and crude extract was kept until used (Rois et al., 1987).

Administration Doses

The doses prepared from the extracted material with a concentration of (85mg/kg body weight) this aqueous extracts, administrated daily for 35 days (Shukla et al., 2000).

Treatment of males

Twenty adult male mice (30-36g.) were purchased from Biotechnology Research Centre-Al-Nahrain University and maintained on a 14/10-hour light/dark cycle in the Animal house control and treated mice were provided with feed and water *ad libitum*, there were no differences in feed intake. Males were randomly divided into 2 groups, each composed of 10 mice. The first group was treated with 85 mg/kg body weight of *Salvia officinalis* extract intraperitoneally daily administered for 35 days and the second group was given normal saline as a control group. The males in each group were sacrificed by dislocation of cervical vertebrate. Sperms were obtained from the two caudae of epididymides by mincing in 500 µl tissue culture medium-199 (capacitation drop) under oil. Spermatozoa were allowed to disperse for 2–3 min at room temperature. A sample of sperm suspension for ICSI was taken immediately after sperm dispersion. For IVF, a sample of sperm suspension was taken after 1.5 h of capacitation. Before capacitation treatment, sperms taken to measured the testes, sperms motility, percentage of dead sperm and abnormalities of sperm were recorded (Monika and Ward, 2005).

Treatment of females

Thirty adult female mice (30-36 g) were purchased from Biotechnology Research Centre-Al-Nahrain University and maintained on a 14/10-hour light/dark cycle in the Animal house control treated mice were provided with feed and water *ad libitum*, there were no differences in feed intake. Female were treated with super ovulation regimen with injections of 5 IU of eCG and 5 IG of hCG given 48 h apart. Oviducts were removed 14–15 h after the injection of hCG and placed in PBS in a Petri dish containing 1ml PBS, then the oviducts were isolated. Cmulus oocyte complex was released from the ampullary region of each oviduct into oil by rupturing the oviduct with the aid of a 25-gauge needle. The oviduct was discarded and the cumulus-oocyte complex moved into the fertilization drop. For IVF and ICSI, the cumulus-oocyte complexes were released from the oviducts into 0.1% bovine testicular hyaluronidase (300 units/mg) in Hepes-buffer Earle's medium to disperse cumulus cells. The cumulus-free oocytes were washed with Hepes- buffer Earle's medium and used immediately for IVF and ICSI (Monika and Ward, 2005).

Microscopic examination

Sperms were assessed according to world health organization Laboratory manual (WHO, 1999) for Motility, percentage of dead/live sperm and abnormalities. The oocytes are then examined under the inverted microscope to assess the maturation stage by observing the presence of a germinal vesicle, germinal vesicle breakdown, and the extruded first polar body. Metaphase II oocytes are identified by the presence of the extruded first polar body.

In vitro fertilization (IVF)

The method for sperm capacitation and IVF using TCM-199 medium has been described elsewhere (Quinn et al., 1982). Briefly, 200-ml drops of TCM-199 medium (fertilization drops) were overlaid with mineral oil in a plastic culture dish (diameter, 60 mm) and equilibrated overnight at 37ºC in a humidified atmosphere of 5% CO_2 in air. The volume of sperm suspension added to the fertilization drop was dependent on the concentration of spermatozoa after dispersion in the capacitation drop. Generally, 10 ml of sperm suspension from the capacitation drop were added to each fertilization drop to give the final concentrations of approximately 2×10^6 sperm/ml. The contents of four oviducts were released into each fertilization drop. After gamete coincubation for 4 h, the oocytes were washed several times with Hepes- buffer Earle's medium followed by at least one wash with buffer Earle's medium. Only morphologically normal oocytes were selected for culture.

Intracytoplasmic sperm injection (ICSI)

The ICSI was carried out as described recently by Szczygiel and Yanagimachi (2003). Briefly, a small drop of sperm suspension was mixed thoroughly with an equal volume of Hepes- buffer Earle's medium containing 12% (w/v) polyvinyl pyrrolidone immediately before ICSI, which was performed using the micromanipulators. A single motile spermatozoon was drawn, tail first, into the injection pipette and moved back and forth until the head-mid piece junction (the neck) was at the opening of the injection pipette. The head was separated from the mid piece. After discarding the mid piece and tail, the head was redrawn into the pipette and injected immediately into an oocyte. The ICSI was done in Hepes- buffer Earle's medium within 1–2 h after oocyte collection. Only motile spermatozoa were used for injection in the present study, because earlier observations indicated that the incidence of abnormal sperm karyotypes increased when spermatozoa were not selected for motility. Sperm-injected oocytes were transferred into TCM-199 medium and cultured at 37ºC. The oocytes were examined approximately 6 h after ICSI for survival and activation.

Embryo culture

After IVF and ICSI, the oocytes were placed in 50-ml drops of TCM-199 medium pre-equilibrated overnight with humidified 5% CO2 in air. The culture drops were contained in plastic culture dishes and overlaid with mineral oil. The survival of ICSI oocytes was scored 1–2 h after the commencement of culture. The number of 2-cell embryos (fertilized) was recorded after 24 h in culture (Monika, 2005).

Statistical analysis

Statistical analysis was performed to compare two different groups by using Chi-square and ANOVA-test. Statistical significance was determined at $P<0.05$ (Al-Mohammed et al., 1986).

Results and discussion

The results show significant increased in percentage of sperm

motility and decreased in percentage of dead/live sperms and percentage of sperm abnormalities in group treated with *Salvia officinalis* as compared with control group (Table 1).

Table 1. Effects of *Salvia officinalis* on spermatozoa activity, percentage of dead sperms and abnormalites in mice

Group	Sperm motility % Mean ± SE	Dead sperm % Mean ± SE	Sperm abnormalities % Mean ± SE
Control	72.31 ± 3.92[a]	26.23 ± 2.94[b]	24.33 ± 2.91[c]
Treated with *Salvia officinalis*	86.43 ± 6.54[a]	17.50 ± 3.01[b]	13.39 ± 2.04[c]

*a,b,c - the same letters marks statistically significant differences (P<0.05)

Dead sperm and abnormalities. The decreasing in the abnormalities of sperms, head and tail (Figure 1 and 2) especially after 35days of treatment with plant extract occur when the sperms are in the spermatogonia stage and before mitotic division which represent a source of sperms, and this will be agreement with AL-Rubia, (Cohen et al., 1992; Van Steirteghem et al., 1993) in which this extract do not contain any mutagenic agents. Acosta et al., (1988) pointed that these mutagenic agent can induce the abnormalities in sperms head and tail while non-mutagenic agent do not induce these abnormalities In addition to these compounds which detected in *Salvia officinalis* extract may play a role in antioxidant protection against sperm damage (Greco et al., 2005b). This test represent a more sensitive test for detection the mutagenic compounds or the decreasing in the sperms head and tail abnormalities may be returned to that these extracts have protective effectives in germ stem cells (spermatogonia) which act as a source of all sperms (Keskes -Ammar et al., 2003).

Salvia extract improved results after treated with salvia could be due to the antioxidant property of salvia evidenced by restoration of glutathione and LPO levels. Restoration of acid phosphatase level show the role of salvia extract in promoting the stability of cellular, nuclear and organelle membranes (Greco et al., 2005a).

Sperm motility. Oxygen radical generation is known to be detrimental to sperm function, especially motility, through the lipid peroxidation of the membranes. The results of this study confirm effect of *Salvia officinalis* on motility and show that antioxidants have a beneficial effect 72.31% in control and 86.43% in treated group. The decrease in sperm motility occurs in the absence of any detectable decrease in viability, acrosomal integrity. A greater protective effect against lipid peroxidation has been observed in bovine semen samples frozen with vitamin E and then incubated with vitamin E after thawing versus samples incubated without the antioxidant (Beconi et al., 1993). The extract of *Salvia officinalis* restores significantly the glutathione level in the liver, and LPO, acid phosphatase and alkaline phosphates in testis of mice.

Micromanipulation involves mechanical alteration of the oocyte *in vitro* to increase the chance of fertilization of the oocyte by sperm. With micromanipulation, fertilization and pregnancy rates appear to be independent of sperm quality (Cohen et al., 1992), which is the opposite of what has been demonstrated for both IUI and IVF (Acosta et al., 1988). This technique for manipulation has a higher risk of oocyte injury, but overall higher fertilization and pregnancy rates (Van Steirteghem et al., 1993). This study indicates that *Salvia officinalis* extract play a role in IVF and ICSI for increasing of percentage on fertilized oocytes.

In Table 2 the results show increased significantly in percentage of oocyte fertilization Figure 3 by ICSI more than IVF in group treated with *Salvia officinalis* compared with control group. One factor which still remains of the utmost importance for ICSI is sperm integrity. Good quality sperm is essential for the accurate transmission of genetic material. The addition of antioxidants such as ascorbate or tocopherol to sperm preparation medium to restore antioxidant protection has previously been shown to improve DNA integrity in human sperm and to afford protection against induced damage. ICSI was the most invasive technique than IVF and less oocyte survive after that. The result indicates (Table 2) equal (65,97% and 61,53%) percentage after treatment with *Salvia officinalis* extract.

Previous studies have shown that oral antioxidant treatment improves sperm nuclear DNA integrity in men with elevated sperm damage (Greco et al., 2005b; Keskes-Ammar et al., 2004).

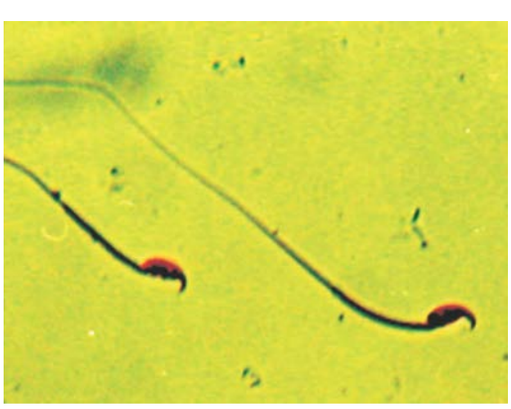

Figure 1. Normal morphological appearance of mice spermatozoa

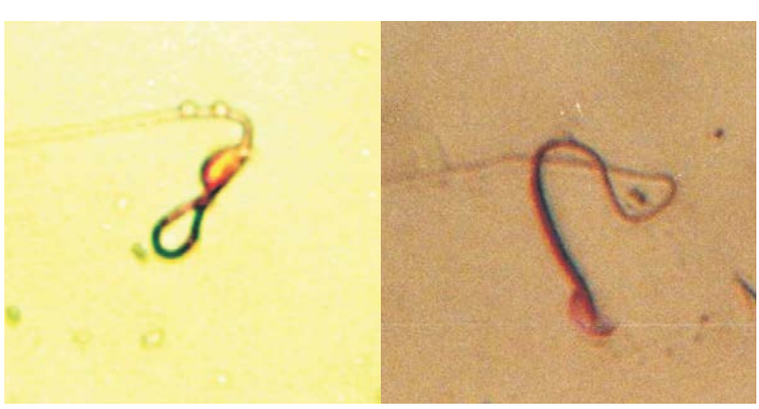

Figure 2. Abnormal appearance of mice spermatozoa

Table 2: Effect of *Salvia officinalis* on percentage of oocyte fertilization by *in vitro* fertilization and ICSI

Group	Maturated oocytes (n)	Oocytes fertilized by IVF (n)	Oocytes fertilized by IVF (%)	Maturated oocytes (n)	Oocytes fertilized by ICSI (n)	Oocytes fertilized by ICSI (%)
Control	91	43	47.25	11	5	45.45
Treated with *Salvia officinalis*	97	64	65.97	13	8	61.53

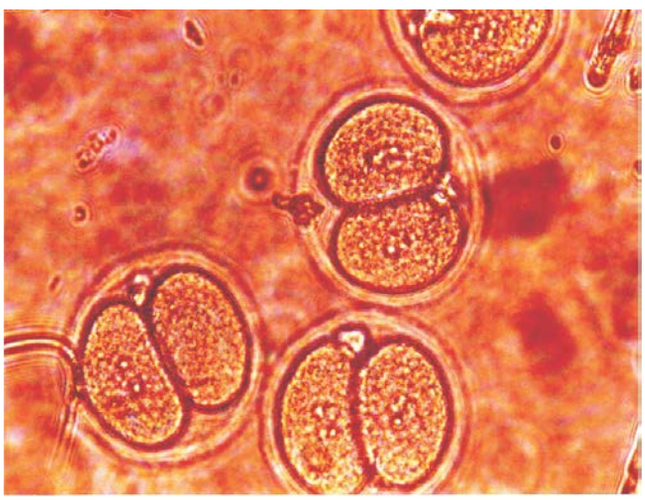

Figure 3. Fertilized eggs demonstrating the two-cells

However, reports concerning the clinical usefulness of antioxidants in the treatment of male infertility are controversial (Agarwal et al., 2004). The data obtained show clearly that ICSI outcomes are markedly improved after the antioxidant treatment in these cases. It is not clear whether at least some improvement would be obtained if a second ICSI attempt with ejaculated spermatozoa were performed also in those cases in which no difference in the extent of sperm fragmentation was detected before and after the oral antioxidant treatment.

Conclusion

This study shows that the leaf extracts of *Salvia officinalis* improved quality and activity of sperm in Albino mice after intraperitoneally treatment, administered for 35 days. The possible impact of this treatment after the IVF and ISCI on oocyte fertility status and on the fertilizing ability of spermatozoa was demonstrated. The leaf extracts of *Salvia officinalis* as tools for increasing percentage of fertilization in assisted reproductive technologies remain to be evaluated.

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The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods, Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

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Simm G, Lewis RM, Grundy B and Dingwall WS, 2002. Responses to selection for lean growth in sheep. Animal Science, 74, 39-50

Books: Author(s) surname and initials, year. Title. Edition, name of publisher, place of publication. Example:

Oldenbroek JK, 1999. Genebanks and the conservation of farm animal genetic resources, Second edition. DLO Institute for Animal Science and Health, Netherlands.

Book chapter or conference proceedings: Author(s) surname and initials, year. Title. In: Title of the book or of the proceedings followed by the editor(s), volume, pages. Name of publisher, place of publication. Example:

Mauff G, Pulverer G, Operkuch W, Hummel K and Hidden C, 1995. C3-variants and diverse phenotypes of unconverted and converted C3. In: Provides of the Biological Fluids (ed. H. Peters), vol. 22, 143-165, Pergamon Press. Oxford, UK.

Todorov N and Mitev J, 1995. Effect of level of feeding during dry period, and body condition score on reproductive performance in dairy cows, IX th International Conference on Production Diseases in Farm Animals, Sept.11 – 14, Berlin, Germany, p. 302 (Abstr.).

Thesis:

Penkov D, 2008. Estimation of metabolic energy and true digestibility of amino acids of some feeds in experiments with muscus duck (Carina moshata, L). Thesis for DSc. Agrarian University, Plovdiv, 314 pp.

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