The Effects of *Aquilaria malaccensis* Leaves Aqueous Extract on Sperm of Sprague Dawley Rats towards Early Embryogenesis

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**ABSTRACT**

**Introduction:** Oxidative stress induced by excessive and unopposed levels of reactive oxygen species in male reproductive system results in impaired sperm quality, fertilization capacity and poor embryo development. Our goal is to assess the potential effects of *Aquilaria malaccensis* (AM) leaves, a plant with strong antioxidant property on early embryo development *in vitro* and embryo quality following fertilization with cyclophosphamide (CP) exposed rat sperm. **Materials and Methods:** Twenty four male Sprague Dawley rats were allocated into eight groups of three rats (n = 3): control, CP only (200 mg/kg), AM only (100 mg/kg, 300 mg/kg and 500 mg/kg) and CP + AM (100 mg/kg, 300 mg/kg and 500 mg/kg). Animals were sacrificed after 63 days of treatment and sperm from caudal epididymis were taken for *in vitro* fertilization (IVF) with oocytes from untreated female. Fertilization, embryo division and embryo morphology were examined at 8 and 48 hours post insemination and compared between groups. Statistical evaluations were performed using Chi-Square test and Fisher’s exact test and \(p\)-value<0.05 was considered significant. **Results:** Administration of AM leave extract at 100 mg/kg/day to normal rats and CP-exposed rats has significantly increased \(p\)-value<0.05) the fertilization rate, early cleavage rate and embryo quality when compared to CP only treated group. However, other groups showed no significant differences. **Conclusion:** Overall, the present results indicate the potential of AM leave extract supplementation to improve the fertility and early embryo development in male rat exposed to CP by inhibiting the oxidative processes and scavenging free radicals.

**KEYWORDS:** *Aquilaria malaccensis*, Cyclophosphamide, *In Vitro* Fertilization, Embryo, Natural antioxidant

**INTRODUCTION**

Oxidative stress (OS) has been widely implicated as one of the important mechanisms in the pathophysiology of male infertility. Under physiological condition, human spermatozoa produces small amount of reactive oxygen species (ROS) to induce capacitation, acrosomal reaction and acquisition of sperm fertilizing ability. However, excessive generation of ROS accompanied by low scavenging and antioxidant levels in semen will lead to induction of OS. The high levels of ROS mainly superoxide anion, hydroxyl radical and hydrogen peroxide will interfere with membrane lipids, proteins and DNA of the sperm and subsequently harm its motility, viability, morphology as well as the
membrane fusion events such as the acrosome reaction and sperm-oocyte fusion. Studies have shown that high levels of ROS were detected in 25-40% of the semen of infertile men, in contrast to low levels in fertile men. Moreover, significant negative correlations have been detected between OS and semen parameters, fertilization rate, embryo development and pregnancy rate.

The origins of ROS in seminal plasma could arise from either endogenous factors including immature or abnormal sperms, infection, varicocele, cancer or exogenous factors such as exposure to drugs, chemicals, heat or radiation, smoking, stress and obesity. Cyclophosphamide (CP), a widely used anticancer and immunosuppressive agent, is well known to induce reproductive toxicity in human as well as rodents as a result of oxidative stress. One of the CP metabolites, acrolein has been proven to increase lipid peroxidation, induce oxidative damage, cytoskeletal abnormalities, and decrease the viability of Sertoli cells in the testis. Furthermore, this toxic alkylating agent also increases the susceptibility of sperm to oxidative attack by alkylating the free thiols on sperm protamines and significantly impairs the chromatin compaction. An increased incidence of oligospermia and azoospermia were observed in male cancer patients treated with CP and some of them even suffered permanent sterility. Apart from that, previous studies on male rats also revealed the potential of CP to cause histological alterations in the testis and epididymis as well as disturbance in gonadotropins and testosterone secretions in blood. CP administration to adult male rats were also reported to adversely affect the offspring by increasing pre- and post-implantation loss, inducing foetal malformations as well as growth retardation. Due to the significant testicular damage and sterility caused by CP, this drug was used to generate sub-fertile rat model in this study and high dose of 200 mg/kg was chosen.

Antioxidants are molecules that act to prevent or intercepting the deleterious effects of free radicals by stabilizing or deactivating them. Three levels of protection offered by antioxidant defence mechanisms include prevention, interception and repair. In order to balance the deleterious ROS effects, seminal plasma and spermatozoa have developed antioxidant systems which comprise of enzymatic antioxidants including superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, and ceruloplasmin; and non-enzymatic antioxidants including albumin, β-carotenes, L-carnitine, glutathione, pyruvate, taurine, hypotaurine, ubiquinol, vitamin C (ascorbic acid), vitamin E (α-tocopherol), and zinc. It has been reported that semen from fertile men has a higher total antioxidant capacity (TAC) than semen from infertile men. To date, the beneficial effects of oral antioxidant therapy in patients with impaired sperm quality have been well documented in several reviews of clinical studies. For example, it has been demonstrated that vitamin E, C and Menevit (combined oral antioxidants) supplementations in male patients undergoing IVF or ICSI treatment have led to significant increase in fertilization rates, implantation rates and pregnancy outcome.

Aquilaria species (agarwood) from the Thymelaeaceae family is one of the most precious plants on earth and has been used in religious, aromatic and medicinal preparation since thousands of years. In folk medicine, its common uses include inflammatory-related ailments such as arthritis, asthma, gout, acting as aphrodisiac and stimulant as well as sedative and carminative agent. Recently, variety of their parts including leaves, skin, seeds, wood and roots were shown to be valuable in medicinal properties. Aquilaria malaccensis (AM) is one of the most common Aquilaria species found in Malaysia and was shown to exhibit potent antioxidant, analgesic, antipyretic, anti-inflammatory, anti-hyperglycemic and antimicrobial activities. Although this plant has been reported to have medicinal purposes towards various kinds of diseases, information on its effect on male fertility and reproduction is very scarce. Therefore, the present study was undertaken to evaluate the possibilities of AM in improving sperm fertilizing ability and subsequent early embryo development in vitro following toxic paternal exposure to cyclophosphamide.

MATERIALS & METHODS

Plant material

The leaves were collected from AM tree plantation in Forest Research Institute Malaysia (FRIM) at Kepong, Selangor. The specimen voucher PIIUM 0296
was prepared and deposited at Natural Medicinal Product Centre, Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM).

**Preparation of the aqueous extract**

The fresh leaves were dried in drying oven at 40 °C and powdered with an electric blender. 150 g of dried leaves powder was soaked in 1500 ml of 40 °C hot distilled water and placed in sonicator for 30 minutes. The hot-water extract was filtered through Whatman No. 1 filter paper and the extraction steps were repeated twice. The resulting filtrates were then freeze-dried and the powder yield was stored at 4 °C in air-tight bottle until further use. The average (W/W) yield was 10%.

**Animals**

Sixty-four Sprague Dawley rats (24 males and 30 females) at 10-12 weeks old with weight between 150-200g were acclimatized for a week before starting the treatment. The animals were kept in polycarbonate cages inside a well-ventilated room with temperature 22 ± 2°C, maintained under standard laboratory conditions with 50 ± 10% humidity and a cycle of 12h light and 12h dark. Standard laboratory animal feed and water were provided *ad libitum*. All ethical themes of studies on animals were considered carefully, and the experimental protocol was approved by the Institutional Animal Care and Use Committee of the International Islamic University Malaysia (IACUC-IIUM) with the reference number IIUM/IACUC-Approval / 2017 (15).

**Experimental protocol**

The male rats were randomly divided into eight groups consisting of three animals each (*n*=3). Group 1 served as normal control and only received distilled water orally. Group 2 received single intraperitoneal injection of CP (200mg/kg) only on day 1 of treatment. Groups 3-5 received supplementation of AM leaves extract daily at concentration of 100 mg/kg/day, 300 mg/kg/day, and 500 mg/kg/day respectively for each group via oral gavage.

**Sperm collection**

The method used for IVF was a modification of that described by Toyoda and Chang.27,28 One day prior to IVF procedures, culture dishes containing drops of fertilization and culture media (LifeGlobal), 30-50 µL each covered with mineral oil were prepared and placed in incubator (37°C, 5% CO₂ in air) overnight to allow them to gas equilibrate. The male rats were euthanized after 63 days of treatment by injection of sodium pentobarbital (60 mg/kg) intraperitoneally and their cauda epididymides were removed. The epididymal sperm were collected by chopping one cauda epididymis in petri dish containing 4 ml of sperm washing media (IrvineScientific). The sperm suspension were left in the CO₂ incubator (37°C, 5% CO₂ in air) for at least 1 hour to allow the sperm to swim up and capacitate before proceeded with IVF. Sperm from cauda epididymis were used because these sperm have gained full maturity and motility. Thus, higher potential for fertilization can be achieved.

**Oocytes collection and insemination**

For collection of ovulated oocytes, female rats were killed on the day of oestrous, which was identified by examination of vaginal smears. The oviducts were removed and kept in handling media (LifeGlobal). The oviducts were then torn open using a dissecting needle and the oocytes were released from within, collected and introduced into the drop of preincubated fertilization media in the culture dishes using the micropipette. Using the tip of pipette, appropriate amount of the preincubated sperm suspension were added to the fertilization media containing the oocytes and the dishes were placed in incubator (37°C, 5% CO₂ in air).

**Embryo evaluation and grading**

About 20-24 hours after insemination, the oocytes were examined for evidence of sperm penetration by means of an inverted phase-contrast microscope. Fertilized oocytes were then washed and transferred into new droplets of culture media and embryo development was assessed again at 48 hours and 72 hours post insemination. The presence of early
embryo cleavage and several morphological parameters of the embryos were evaluated. Cleaved embryos scoring was applied based on the number of cells, the appearance of blastomeres and the presence of cytoplasm defects or fragmentation as described by Baczkowski et al. Grading based on symmetry of blastomere were as follow; Type A: equal size blastomeres; Type B: unequal size blastomere and Type C: defects of cytoplasm. Additionally, grading based on degree of fragmentation includes; Grade I: no fragmentation; Grade II: fragmentation less than 30%; Grade III: fragmentation between 30-50% and Grade IV: fragmentation more than 50%.

### Statistical analysis

Statistical evaluations were performed using Chi-Square test and Fisher’s exact test. SPSS version 21.0 was used for statistical analysis and p-value<0.05 was considered significant in this study.

## RESULTS

### Fertilization rate and early embryo cleavage

Table I presents the fertilization, four-cell and eight-cell cleavage rates of IVF cultures for each group. The rates were calculated as the number of fertilized oocytes (presence of two pro-nuclei), 4-cell stage (number of 4-cells embryos 48 hours post insemination) and 8-cell stage embryos (number of 8 cells embryos 72 hours post insemination) divided by the total number of oocytes observed per group respectively. Significant increase (p<0.05) in the rate of fertilization and 4-cell stage embryo development were seen in the group treated with AM at dose 100 mg/kg (AM-100) compared to the CP only group. Co-treatment of CP exposed rats with AM at same dose (CP+AM-100) also resulted in significantly higher (p<0.05) fertilization rate compared to CP only group. However, other groups have no significant difference in comparison with control and CP group.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of oocytes</th>
<th>Fertilization rate</th>
<th>4-cell rate</th>
<th>8-cell rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33</td>
<td>52% (17)</td>
<td>39% (13)</td>
<td>30% (10)</td>
</tr>
<tr>
<td>CP</td>
<td>30</td>
<td>30% (9)</td>
<td>20% (6)</td>
<td>17% (5)</td>
</tr>
<tr>
<td>AM-100</td>
<td>37</td>
<td>62% (23)</td>
<td>43% (16)</td>
<td>32% (12)</td>
</tr>
<tr>
<td>AM-300</td>
<td>35</td>
<td>37% (13)</td>
<td>23% (8)</td>
<td>17% (6)</td>
</tr>
<tr>
<td>AM-500</td>
<td>33</td>
<td>45% (15)</td>
<td>30% (10)</td>
<td>21% (7)</td>
</tr>
<tr>
<td>CP+AM-100</td>
<td>35</td>
<td>57% (20)</td>
<td>40% (14)</td>
<td>29% (10)</td>
</tr>
<tr>
<td>CP+AM-300</td>
<td>32</td>
<td>28% (9)</td>
<td>19% (6)</td>
<td>16% (5)</td>
</tr>
<tr>
<td>CP+AM-500</td>
<td>33</td>
<td>24% (8)</td>
<td>15% (5)</td>
<td>12% (4)</td>
</tr>
</tbody>
</table>

Data is presented in percentage. Significantly different from CP group, p-value<0.05 (Chi-Square test).

Abbreviation: CP = Cyclophosphamide; AM-100 = Aquilaria malaccensis 100 mg/kg/day; AM-300 = Aquilaria malaccensis 300 mg/kg/day; AM-500 = Aquilaria malaccensis 500 mg/kg/day

### Blastomere structure of embryos

Table III shows the percentage of embryos graded based on symmetry of the blastomeres. There was no significant difference in the structure of the blastomere including type A, type B and type C between all the groups. Descriptively, most of the embryos produced in all groups were under type B (unequal size blastomere) and CP only treated group indicated highest percentage of type C (defects of cytoplasm) embryos.

### DISCUSSION

One of the important determinants for successful fertilization and embryo development in vitro is the sperm quality. Many studies have demonstrated low
fertilization rates, cleavage rates and embryo quality associated with poor semen parameters, indicating a very early onset of paternal effects on embryo development. These significant parameters include reduced sperm concentration, motility and high percentage of morphologically abnormal sperm as a result of impaired spermatogenesis. The quality of DNA in the sperm head can also influence embryo development. Clinical studies have proven that infertile men possess more sperm DNA damage in the ejaculate than do healthy subjects. Using sperm containing high loads of DNA damage during ART has led to poor embryo development and early pregnancy loss.

Table II: The effects of cyclophosphamide (CP) and *Aquilaria malaccensis* (AM) leave aqueous extract on fragmentation degree of cleavage stage rat embryos

<table>
<thead>
<tr>
<th>Group</th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
<th>Grade IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41% (7)</td>
<td>41% (7)</td>
<td>18% (3)</td>
<td>0% (0)</td>
<td>100% (17)</td>
</tr>
<tr>
<td>CP</td>
<td>0% (0)</td>
<td>75% (6)</td>
<td>25% (2)</td>
<td>0% (0)</td>
<td>100% (8)</td>
</tr>
<tr>
<td>AM-100</td>
<td>48% (11)†</td>
<td>48% (11)†</td>
<td>4% (1)</td>
<td>0% (0)</td>
<td>100% (23)</td>
</tr>
<tr>
<td>AM-300</td>
<td>54% (7)†</td>
<td>38% (5)</td>
<td>8% (1)</td>
<td>0% (0)</td>
<td>100% (13)</td>
</tr>
<tr>
<td>AM-500</td>
<td>50% (7)†</td>
<td>43% (6)</td>
<td>7% (1)</td>
<td>0% (0)</td>
<td>100% (14)</td>
</tr>
<tr>
<td>CP+AM-100</td>
<td>45% (9)†</td>
<td>30% (6)</td>
<td>20% (4)</td>
<td>5% (1)</td>
<td>100% (20)</td>
</tr>
<tr>
<td>CP+AM-300</td>
<td>33% (3)</td>
<td>67% (6)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>100% (9)</td>
</tr>
<tr>
<td>CP+AM-500</td>
<td>14% (1)</td>
<td>43% (3)</td>
<td>43% (3)</td>
<td>0% (0)</td>
<td>100% (7)</td>
</tr>
</tbody>
</table>

Data is presented in percentage.

Table III: The effects of cyclophosphamide (CP) and *Aquilaria malaccensis* (AM) leave aqueous extract on blastomeres structure of cleavage stage rat embryos

<table>
<thead>
<tr>
<th>Group</th>
<th>Types A</th>
<th>Types B</th>
<th>Types C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12% (2)</td>
<td>59% (10)</td>
<td>29% (5)</td>
<td>100% (17)</td>
</tr>
<tr>
<td>CP</td>
<td>13% (1)</td>
<td>50% (4)</td>
<td>38% (3)</td>
<td>100% (8)</td>
</tr>
<tr>
<td>AM-100</td>
<td>26% (6)</td>
<td>61% (14)</td>
<td>13% (3)</td>
<td>100% (23)</td>
</tr>
<tr>
<td>AM-300</td>
<td>8% (1)</td>
<td>77% (10)</td>
<td>15% (2)</td>
<td>100% (13)</td>
</tr>
<tr>
<td>AM-500</td>
<td>21% (3)</td>
<td>64% (9)</td>
<td>14% (2)</td>
<td>100% (14)</td>
</tr>
<tr>
<td>CP+AM-100</td>
<td>20% (4)</td>
<td>55% (11)</td>
<td>25% (5)</td>
<td>100% (20)</td>
</tr>
<tr>
<td>CP+AM-300</td>
<td>33% (3)</td>
<td>56% (5)</td>
<td>11% (1)</td>
<td>100% (9)</td>
</tr>
<tr>
<td>CP+AM-500</td>
<td>14% (1)</td>
<td>71% (5)</td>
<td>14% (1)</td>
<td>100% (7)</td>
</tr>
</tbody>
</table>

Data is presented in percentage.

Abbreviation: CP = Cyclophosphamide; AM-100 = *Aquilaria malaccensis* 100 mg/kg/day; AM-300 = *Aquilaria malaccensis* 300 mg/kg/day; AM-500 = *Aquilaria malaccensis* 500 mg/kg/day

In this study, acute exposure of male rats to high dose of CP was expected to impair sperm parameters and consequently affect the IVF outcome. Cyclophosphamide is known to induce both structural and functional sperm impairment by exerting direct toxic effect on DNA of the male germ cells and causing peroxidation of polyunsaturated fatty acids in plasma membranes of spermatozoa by generation of free radicals. These sperm defects are associated with low sperm fertilizing capacity and poor embryo development if fertilization occurs, due to DNA damage transmission to zygote. A
number of studies have revealed that paternal exposure to CP causes a decrease proliferation in cleavage-stage embryos, a laggard cell division pattern and a reduction in blastomere number and cell to cell contacts.\textsuperscript{38} In our study, the major findings showed that concurrent administration of AM leaves extract at 100 mg/kg to male rats after CP exposure significantly improve the fertilization rate and reduce embryo fragmentation following IVF. This indicates that AM leaves extract at 100 mg/kg has the potential to reduce free-radicals mediated sperm damage induced by CP and improve the sperm DNA integrity. Thus, sperm structure and function can be enhanced and more fertilization and good embryo development can be achieved.

To the best of our knowledge, the current study is the first to investigate the effects of Aquilaria species specifically Aquilaria malaccensis (AM) on male reproduction and the resulting IVF outcome. Increasing evidences support the fact that AM is advantageous where free radicals are known to play a predominant role in toxicity such as cancers and diabetes.\textsuperscript{39} Studies on different extracts of A. malaccensis leaves including hexane, methanol, ethyl-acetate, and water showed that these extracts exhibited strong antioxidant and free radical scavenging activity. Moreover, it was reported that highest total phenolic content were manifested by water extract of the dried AM leaves.\textsuperscript{26,40-42} Administration of A. malaccensis bark extract to mice via gavage was also demonstrated to increase physical fitness and sexual activities such as kissing vagina and mounting as well as the sperm parameters including sperm count and motility.\textsuperscript{43} It was also reported that A. malaccensis have cytotoxic activity towards cancer cells line and can serve as an alternative treatments for several types of cancer. Cytotoxic measure of A. malaccensis oil showed high cytotoxic activity towards colon (HCT116) cancer cell line and able to inhibit up to 99% of the cancer cells.\textsuperscript{44} The essential oil was also observed to possess anticancer activity towards MCF-7 breast cancer cells, where it showed cumulative effect of the cell killing, inhibition of the cell attachment and causing the cells to detach.\textsuperscript{45} These important pharmacological properties are attributed to the presence of many bioactive chemical constituents such as alkaloids, tannins, phenols, terpenoids, quinones and flavonoids, which are known to possess high antioxidant activity and provide protection against cells damage and substantially reduce the risk of developing chronic disease.\textsuperscript{26,41,42}

However, our study indicates that AM at dose 300 mg/kg and 500 mg/kg showed no improvement in fertilization, cleavage rates or embryo grading when administered to CP treated rats. Generally, AM leave aqueous extract administration is considered safe and the AM dosage should not cause any negative effects on sperm and embryo. Previous study indicated that AM leave extract administration up to 520 mg/kg daily for 90 days in male mice does not cause toxic symptoms and is safe for consumption.\textsuperscript{46} The LD\textsubscript{50} of the leaves extract was also found to be above 2000 mg/kg.\textsuperscript{47} Moreover, significant increase in grade 1 embryos in IVF of normal rats supplemented with AM at dose 300 mg/kg and 500 mg/kg indicates the potential benefits of AM on embryo development at this dosage. Possible factors that may explained the insignificant effects of AM at these doses include improper handling of gametes and embryo during IVF procedures or environmental factors during embryo culture such as 'hypoxic' in vitro environment, visible light or infection. These factors can cause injury to the cells, induce free radical generation and subsequently impair the fertilization process and embryo division.\textsuperscript{6,48} Therefore, replication of the study with higher sample size is needed to prove the validity of the findings. In addition, evaluation of AM leaves extract toxicity in vitro using cell lines can be done to optimize the toxicity studies.

**CONCLUSION**

In conclusion, the findings of our study indicate that AM co-administration following paternal CP exposure has the potential to protect the male gametes from cellular and structural damage, improve the sperm fertilizing capacity and subsequent embryo development by inhibiting the oxidative processes and scavenging free radicals. However, additional research work is needed to further elucidate the mechanism of protective role of AM in CP induced-toxic manifestation and to investigate the most efficient compound responsible for the therapeutic effects.

**DECLARATION OF INTEREST**

The authors declare no conflict of interest.
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REFERENCES


44. Ibrahim AH, Al-Rawi SS, Abdul Majid AMS, et al. Separation and fractionation of Aquilaria Malaccensis oil using supercritical fluid extraction and the cytotoxic properties of the...