THE PROPHYLACTIC EFFECT OF NIGELLA SATIVA AGAINST CYCLOPHOSPHAMIDE IN THE OVARIAN FOLLICLES OF MATURèD ADULT MICE: A PRELIMINARY STUDY

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ABSTRACT

This study aims to assess follicle preservation in mature female mice with the provision of Nigella Sativa oil against effects of cyclophosphamide which can cause ovarian follicular loss. Forty-eight ICR mice aged 18 weeks were divided into three groups: group I = control, group II = cyclophosphamide-alone (50mg/kg) for 5, 10, 15 and 20 days; and group III = pre-treated with different doses of Nigella sativa oil (0.2ml, 0.5ml and 1.0ml/100g) for 5 days, all by intraperitoneal injection. The histology and the total number of ovarian follicles were studied. Results show that the number of primordial follicles following exposure to cyclophosphamide was significantly reduced (36.33 ± 5.86) as compared to the controls (86.67 ±32.52) at day 10 (p<0.05). There was also a significant reduction in the mean number of normal primary and secondary follicles (p<0.001), mean ovarian diameters (p<0.05) and an increased vacuolation with irregular distribution of granulosa cells. The numbers of normal primary and secondary follicles including ovarian diameters were significantly increased in the use of Nigella sativa as opposed to effects seen in controls and cyclophosphamide-alone groups at day 5. This study seems to suggest a prophylactic property of Nigella sativa in the reproductive system of female mice.

Keywords: Nigella sativa oil, Cyclophosphamide, Ovarian follicles, Mature mice, Infertility

INTRODUCTION

The advancement in the field of oncology and continued development in treatment modalities has increased the awareness on the long term effects of chemotherapy treatment on the reproductive potential of cancer survivors. It is increasingly noted that a high number of adult survivors of childhood cancer malignancies were not aware of the risk of infertility and relevant late effects (Hess et al., 2011). Chemotherapeutic agents have been shown to affect female fertility by destroying the ovarian follicles and inducing ovarian damage that can lead to infertility. Ovarian damage occurs in all age groups with older females appearing to be more affected as they have a smaller ovarian follicular reserve (Meirow et al., 1999; Meirow and Nugent, 2001).

Cyclophosphamide (CPA) is one of the most damaging alkylating agents that can cause oxidative stress due to the over-production of reactive oxygen species (ROS) (Damewood and Grochow, 1986; Pryor et al., 2000; Meirow and Nugent, 2001; Mitchell et al., 2003; Alenzi et al., 2010). It affects the DNA of replicating cells and rapidly multiplying cells especially in the gonads and pituitary which results in miscoding, cross-linking and DNA breakage by transferring alkyl groups to the guanine compound of the DNA (Becker and Schoneich, 1982).

In the females, the prime concern now is that cyclophosphamide can induce depletion in the primordial follicular (PMF) reserve. Since PMF pool is non-renewable, older women treated with chemotherapy have a higher incidence of ovarian failure when the chemotherapeutic agent destructs an already low follicular reserve needed to sustain ovarian function (Kumar et al., 1972; Gosden and Faddy, 1994).

A potential approach that can interfere with cyclophosphamide-induced toxicity is to lower the induced oxidative stress. Nigella sativa, from the Ranunculacea family, has been one of the most widely used herbal medicines for the treatment of various diseases. The pharmacological properties of the oil have been reported to include anti-inflammatory, anti-cancer, anti-diabetic, anti-microbial, anti-histaminic, anti-infertility and hypotensive effects (Mukhallad et al., 2009; Alenzi et al., 2010). Thymoquinone is the active compound of the essential oil with anti-oxidative effect that works as a scavenger of various radical oxygen species including superoxide radical anion and hydroxyl radicals through different mechanisms (Mansour et al., 2002; Badary et al., 2003; Mahgoub 2003). Thymoquinone was reported to re-establish spermatogenesis after testicular injury caused by chronic toluene exposure in rats (Kanter, 2011).

A recent study by Arak and Assi (2011) reported that adult rats exposed to 10mg/kg of lead acetate caused a significant reduction on ovarian function and the treatment of 100mg/kg of Nigella sativa caused...
significant enhancement on the reproductive function with a decrease in number and diameter of Graafian follicles in comparison to the normal structure.

The role of *Nigella sativa* in the preservation of ovarian structure and function has not been explored in detail. The present work was conducted to study the possible prophylactic effect of *Nigella sativa* oil (NSO) on ovarian injuries as expressed by the loss of PMF population and increased follicle degeneration induced by the anticancer drug cyclophosphamide.

**MATERIALS AND METHODS**

Forty eight ICR mature adult female mice, aged 18 weeks were divided into three main experimental groups (n=6/group). **Figure 1**: group (i) = vehicle-treated control, group (ii) = CPA alone and group (iii) = NSO six hours prior to CPA treatment. In group i, control mice were injected with normal saline. Mice of group II were given a single intraperitoneal injection of cyclophosphamide (Sigma-Aldrich) at a dose of 50mg/kg body weight. This dose was chosen based on a previous dose-response study of Meirow et al., 1999. Observation of effects was done at four different time intervals; days-5, 10, 15 and 20 which represents a different stage of follicular growth at the time of exposure to cyclophosphamide (Meirow et al., 2001). All mice in group ii received cyclophosphamide injection on the same day. In group iii, mice were divided into three different sub-groups and pre-treated intraperitoneally with different doses of *Nigella sativa* oil; 0.2ml/100g, 0.5ml/100g and 1.0ml/100g body weight, 6 hours before single cyclophosphamide injection at 50mg/kg. *Nigella sativa* oil treatment was continued on every other day for 5 days. Observation of effects was done on day 5 post treatment.

At the end of each exposure period, mice were euthanized by cervical dislocation. The ovaries were excised and trimmed free of fat before immersion in the fixative solution. Ovaries were fixed with 4% formaldehyde overnight, dehydrated in ethanol, embedded in paraffin and serially sectioned at 6 μm. The tissues were stained with haematoxylin and eosin.

Ovarian follicles were counted in every section using a light microscope at a magnification of 400X. Follicles were classified into four types based on the classification of Erickson (2003): (i) Primordial - characterized by an oocyte surrounded by a single layer of flattened cells; (ii) Primary - characterized by a single layer of cuboidal pre-granulosa cells; (iii) Secondary - characterized by 2 to 5 complete layers of granulosa cells and (iv) Graafian - containing cavity occupying most of the total follicular volume. Only follicles with a visible nucleus in the oocyte were considered for counting to avoid duplicate counts of a follicle. Follicles that contained an intact oocyte and intact granulosa cells were classified as normal. Follicles were classified as degenerated when they contained ruptured oocyte nuclei, shrunken ooplasm and disorganized granulosa cells. The diameters of the ovary were further measured at a magnification of 40X by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present. Ovary was divided into sections and follicles distribution was counted using Olympus Analysis Image Processing Software which will mark the counted follicles to avoid duplication.

Statistical analysis was done with SPSS 18.0 software. All the values of primordial follicles, ovarian diameters and the distribution of normal and degenerated follicles were expressed as mean ± standard deviation (S.D). Inter-group variation was measured by one-way analysis of variance (ANOVA) and Tukey’s test to evaluate the significant differences between the groups. p ≤0.05 was considered to be statistically significant.

Work done in this study received the ethical approval from the Ethics Committee of the Faculty of Medicine of the International Islamic University Malaysia (Ref: IIUM/305/20/4/10).

**RESULTS**

A. **Histological changes of the ovarian follicles:**

Control ovary showed the presence of normal ovarian architecture in the primordial (I), primary (II) and secondary (III) follicles. The oocyte (O) was surrounded by a single or two layers of granulosa cells (G). The oocyte was separated from the surrounding follicular cells by a well developed glycoprotein layer called zona pellucida (ZP). The outermost layer of follicular cells rests on a well defined basement membrane (BM) that separates it from the ovarian stroma. At the periphery, the connective tissue stroma surrounding the follicle begins to condense and form a theca folliculi layer (TL). Theca layer is usually distributed out of the healthy granulosa basement membrane in one, or more rarely, two continuous layers (Fig. 2).

Some injury was observed in the CPA group including the disruption of intercellular contacts among granulosa cells and the oocyte of primary and secondary follicles at day 5 (Fig. 3). These follicles also exhibited numerous cytoplasmatic vacuoles, abnormal shapes of the granulosa cells, some with absent or undeveloped zona pellucida and oocyte, as well as vacuolated oocyte with nuclear shrinkage.

NSO-treated groups showed an improved histological appearance in the CPA-exposed mice. The morphology of the follicles and the structure of the oocytes were well preserved, similar to the control group (Fig. 4).

The CPA-exposed groups of 15 and 20 days showed less signs of degeneration and injury in pre-antral
(primary and secondary) follicles when compared to that in the groups of 5 and 10 days.

B. The total numbers of primordial follicle (PMF): At 5 days of exposure, although there were differences in the mean number of PMF in the cyclophosphamide groups pre-treated with NSO as compared to the cyclophosphamide alone group, the differences were not statistically significant. The One-Way ANOVA test showed significant differences ($p<0.05$) between the different time points of cyclophosphamide exposure and the total number of PMF in the ovaries (Fig. 5). Subsequent post-hoc analysis (Tukey test) suggested that the mean number of PMF in the ovaries of mice exposed to 50mg/kg cyclophosphamide was significantly reduced at day 10 (36.33 ± 5.86), day 15 (23.67 ± 9.07) and day 20 (12.00 ± 1.00) when compared to the control group (86.67 ±32.52). The total number of PMF reduces by about half (58.13%) after 10 days of cyclophosphamide exposure.

C. The total number of normal and degenerated follicles at different follicular stages: A One-Way ANOVA was conducted to explore the impact of cyclophosphamide and NSO on the total distribution of normal and degenerated primary, secondary and Graafian follicles after 5 days of exposure. There was a significant difference in the total number of normal and degenerated primary and secondary follicles between different treatment groups ($p<0.001$). Post-hoc comparison using the Tukey test indicated that the mean distribution of degenerated primary (10.71±4.42), secondary (10.43±3.55) and Graafian follicles (4.71±2.75) was significantly increased in cyclophosphamide-alone group in comparison to the control group (1.60±1.23, 6.30±1.95 and 1.90±0.74 respectively) (Table 1). In contrast, the mean distribution of degenerated primary and secondary follicles was significantly decreased in the three groups pre-treated with NSO. Moreover, animals pre-treated with NSO showed higher mean of normal primary and secondary follicles compared to control and cyclophosphamide-treated groups. No significance differences were observed in the total number of normal Graafian follicles in all treatment groups.

D. Morphometrical analysis: The study revealed a significant reduction in the mean ovarian diameters of the cyclophosphamide-treated groups observed at 15 and 20 days; 674.37±39.12µm and 417.43±14.51µm respectively ($p<0.05$), compared to an average of 1055.56±35.32 m in the control group (Table 2). Cyclophosphamide groups that were pre-treated with 0.5ml and 1.0ml/100g of NSO for 5 days showed a significant higher ovarian diameters (1113.10 ± 14.68µm and 1215.70 ± 14.50µm, respectively, $p<0.05$) compared to the group that received cyclophosphamide alone (919.83 ± 96.43µm).

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**Figure 1:** The schematic representation of the grouping criteria and treatment regimens.
Table 1. Mean ± SD total distribution of normal and degenerated follicles after cyclophosphamide and *Nigella sativa* oil exposures at 5 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Primary Follicles</th>
<th>Secondary Follicles</th>
<th>Graafian Follicles</th>
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<tr>
<td></td>
<td>Normal</td>
<td>Degenerated</td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>3.20 ± 1.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.60 ± 1.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.10 ± 2.92&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>CPA alone</td>
<td>3.71 ± 2.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.71 ± 4.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.43 ± 0.54&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPA + NSO 0.2ml/100g</td>
<td>9.75 ± 1.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.75 ± 1.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.25 ± 2.32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPA + NSO 0.5ml/100g</td>
<td>6.50 ± 4.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75 ± 1.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.88 ± 3.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPA + NSO 1.0ml/100g</td>
<td>6.43 ± 3.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.14 ± 3.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.86 ± 2.34&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> No significant different between groups.  
<sup>b</sup> Significantly different between groups, p<0.05.  
<sup>c</sup> Significantly different between groups, p<0.001.

Table 2: Mean ± SD diameters of the ovaries in the control, cyclophosphamide alone and cyclophosphamide co-treated with *Nigella sativa* oil groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ovarian Diameters (μm)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1055.56 ± 35.32</td>
</tr>
<tr>
<td>Cyclophosphamide 5 days</td>
<td>919.83 ± 96.43</td>
</tr>
<tr>
<td>Cyclophosphamide 10 days</td>
<td>931.30 ± 86.32</td>
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<tr>
<td>Cyclophosphamide 15 days</td>
<td>674.37 ± 39.12&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclophosphamide 20 days</td>
<td>417.43 ± 14.51&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Nigella Sativa</em> oil + cyclophosphamide 0.2ml/100g, 5 days</td>
<td>907.73 ± 34.02&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Nigella Sativa</em> oil + cyclophosphamide 0.5ml/100g, 5 days</td>
<td>1113.10 ± 14.68&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Nigella Sativa</em> oil + cyclophosphamide 1.0ml/100g, 5 days</td>
<td>1215.70 ± 14.50&lt;sup&gt;†&lt;/sup&gt;</td>
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<sup>*</sup>Significantly different from control groups, p<0.05.  
<sup>†</sup> Significantly different from cyclophosphamide 5-days group, p<0.05.

**Plate-I.** Photomicrographs of mice ovary with Haematoxylin & Eosin preparation

**Fig. 2:** Light microscopy of ovarian tissue in control group using haematoxylin and eosin staining. The normal morphology of the ovarian follicles; primordial (I), primary (II) and secondary (III), showing the oocyte (O), granulosa cells (G), zona pellucida (ZP), basement membrane (BM) and theca layer (TL) (H&E, 400X, scale bar 200 μm).
Fig. 3: Light microscopy of ovarian tissue in CPA group (5 days) using haematoxylin and eosin staining showing primary follicle (A) with severe ovarian damage and ruptured oocyte and secondary follicle (B) with disruption of the granulosa cells and absence of oocyte (C) (H&E, 400X, scale bar 200 μm).

Fig. 4: Light microscopy of ovarian tissue in group pre-treated with 0.2ml/100g NSO 6 hours before CPA exposure using haematoxylin and eosin staining showing improved histological appearance of the primordial follicles (P), primary follicles (I) and secondary follicles (II) with intact granulosa cells (GC), prominent oocytes (O), well developed theca layer (TL) and zona pellucida (ZP) (H&E, 400X, scale bar 1000 μm).

Fig. 5: Total number of primordial follicles (PMF) in ovaries of mice exposed to 50mg/kg cyclophosphamide (CPA) at 5, 10, 15 and 20 days compared to the control group (Mean ± SD). PMF loss was significant in groups exposed to CPA at 10, 15 and 20 days as compared to the controls. The total number of PMF reduces by about half (58.13%) at 10 days of CPA exposure. *Significantly different from control groups, p<0.05
DISCUSSION

This study reported the potential prophylactic effects of NSO and the improvement of the survival of mouse ovarian follicles against ovarian toxicity induced by cyclophosphamide. In the present study, treating mice with cyclophosphamide revealed significant histological alterations including atrophy of the ovarian follicle cells, degeneration of the interstitial tissue as well as disruption of the intercellular contacts among granulosa cells. These alterations were more prominent in animals exposed to the anticancer drug for 5 and 10 days. The histological changes in ovarian tissue may be due to hormonal changes caused by cyclophosphamide toxicity. It is well-established that gonadotropins are critical to complete follicular development (Cecconi et al., 2004). FSH supplementation in vitro is useful in driving initial follicle growth by exerting a positive effect on follicle survival and oocyte quality in mouse (Mitchell, Kennedy and Hartshorne, 2002; Adriaens, Cortsrindt and Smitz, 2004). A study by Notolla et al. (2011) reported that the follicles cultured in fetal calf serum alone without FSH supplementation did not form the antral cavity and displayed low differentiation. However, 80% of FSH-treated follicles formed the antral cavity and showed various ultrastructural markers of maturity.

Morphometrical analysis of the PMF number provides information about ovarian toxicity caused by chemotherapy agent more directly rather than reproductive performance i.e. ovulation, mating and pregnancy rates (Meirrow et al., 1999). The present data showed that cyclophosphamide depletes up to 58.13% of the PMF reserve after 10 days of cyclophosphamide exposure. This result is in agreement with a study by Farokhi et al. (2007) which reported that cyclophosphamide administration of 75mg/kg destroyed more than 50% of PMF pool. A study by Meirrow et al. (1999) reported that significant damage to the PMF population resulted even following administration of low doses of cyclophosphamide (20mg/kg). Moreover, a xenograft model used to illustrate the impact of chemotherapy drugs on human primordial follicular reserve reported that animals administered with a single dose of 200mg/kg cyclophosphamide showed a 12% reduction in the PMF density by 12 hours following treatment (p<0.05) and significantly increased in follicle loss at 24 hours (53%, p<0.01). The percentage of follicular loss was peaked at 48 hours (93%, p<0.0001) (Oktem and Oktay, 2007). Animal studies have shown clear evidence that cyclophosphamide causes injury to germ cells. Ovarian function depends on the follicular reserve as they sustain the ovarian function. Thus depletion of PMF reserve indicates ovarian failure which can result in infertility (Aguilar-Mahecha et al., 2002).

Morphometrical parameter such as diameter of the ovary can also give information about the ovarian damage degree as a consequence of germ cell death. Our results showed the diameter of ovaries in cyclophosphamide-treated mice was significantly decreased compared to the controls. Similar findings was reported by Farokhi et al. (2007) indicating cyclophosphamide administration causes reduction in diameter and size of mouse ovary as well as decreased thickness of the endometrium. In general, massive germ cell loss caused by anticancer drugs is followed by significantly lower ovarian diameter and ultimately ovarian atrophy (Kuhajda, Haupt, Moore and Hutchins, 1982). Furthermore, since cyclophosphamide is an antimitogenic agent, it causes reduction in mitotic division in fast dividing cells such as endometrial cells. Therefore, this process will also brings reduction in endometrium wall as well as uterine thickness in the test group.

Assessment of other follicular distribution at different stages of growth revealed that there was significant reduction of normal primary and secondary follicles in the cyclophosphamide-treated group compared with the control group. This indicates that injection of cyclophosphamide brings decrement in normal follicular distribution in the test group. In agreement with this result, the previous studies reported that the mean number of secondary, tertiary follicles and corpora lutea also showed significant reduction in the cyclophosphamide-treated group (Farokhi et al. 2007). The differences of Graafian follicles in the control and cyclophosphamide-treated group was however not significant. Other studies on radiotherapy revealed that the growing oocytes are relatively resistant to anticancer agent compared to PMF.

Toxicity related to anticancer drugs is usually associated with the over-production of reactive oxygen species (ROS) that cause oxidative stress (Mitchell et al., 2003). Oxidative stress has been implicated in a number of different reproductive scenarios such as endometriosis, folliculogenesis, oocyte maturation, hydrosalpingeal fluid, necrozoospermia, asthenozoospermia and sperm DNA damage (Guerin et al., 2001). In the context of female infertility, oxidative stress has been poorly characterized (Agarwal and Allamaneni, 2004). Nevertheless, oxidative stress in the female reproductive system has been demonstrated to correlate with fertility. Markers of oxidative stress in follicular fluid such as lipid peroxidation, total antioxidant capacity and superoxide dismutase activity are strongly correlated with oocyte fertilization and pregnancy rates following IVF (Pasqualotto et al., 2004). A decrease in their total antioxidant capacity may lead to oxidative stress.

Therefore, dietary antioxidants become critical under conditions of increased oxidative stress in maintaining a desirable oxidant-antioxidant balance. In this study, when NSO was administered to the animals together with cyclophosphamide, it improved the histopathological and morphometrical changes induced
by cyclophosphamide in the ovary. The histological structures of the primary, secondary and graafian follicles derived from the groups of cyclophosphamide pre-treated with NSO were more preserved than that achieved from cyclophosphamide-alone group. The combination may also protect the ovarian follicles from the alkylating effects of cyclophosphamide as the number of degenerated follicles was lowered in the three groups pre-treated with NSO compared to cyclophosphamide-treated group.

The combination of NSO with cyclophosphamide seemed to suggest that the antioxidative properties of NSO may have protected the follicular cells from cyclophosphamide which is known to cause the over-production of ROS (Mortensen et al., 2010). This suggestion is in agreement with previous study which reported the improvement of the survival follicles and beneficial effects of Nigella sativa seeds on the morphology and ovarian function of rats against the toxicity effects induced by lead acetate (Arak & Assi, 2011). This study reported the effect of Nigella sativa that resulted in decreasing the ovarian weight to body weight ratio compared to the lead acetate-treated group. The group that received combination treatment of Nigella sativa and lead acetate also showed a decrease in the size of Graafian follicle, a decrease in the number of primary, secondary and Graafian follicles as well as normal structure of the Graafian follicle.

In contrast to our observations, Yadav and Agarwal (2011) reported that the ovarian follicles showed degenerative changes following administration of aqueous extract of Nigella sativa. This dissimilarity in result is due to differences in the treatment regimen of which the rats in that study were administered with a high concentration of Nigella sativa extract (200mg/kg body weight) for the duration of 40 days, without exposure to anticancer drug. There are controversial reports regarding the safety of Nigella sativa. Its oil was safe when given orally to rats (LD50 of 28.8ml/kg) (Zaoui et al., 2002) and oral thymoquinone was also found to be quite safe (LD50 of 2.4 g/kg) (Badary et al., 1998). Zaghlol et al. (2012) reported rats that received large doses of NSO (15 and 25ml/kg) for 1 month has toxic effects on the histological structure of the kidney and liver and therefore concluded that NSO should be used in proper doses.

In conclusion, our observations in correlation with histological and morphometrical analysis showed that prophylactic treatment of mouse ovarian tissue to Nigella sativa oil before cyclophosphamide exposure has induced significant protection on the fine structure of follicles. Also the survival rates of normal follicles in these groups are higher than cyclophosphamide-treated group thus the integrity of mouse ovarian is less likely to be affected by the anticancer drug.

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