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Effect of black cumin (*Nigella sativa*) seeds extract in Tris egg yolk citrate extender on oxidative stress parameters of cryopreserved Surti buck semen

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Abstract
The present study was conducted to examine effect of Black cumin (*Nigella sativa*) seeds extract in Tris egg yolk citrate extender on oxidative stress parameters of cryopreserved Surti buck semen. Total 64 semen ejaculates were collected from four Surti buck (16 ejaculates/buck) twice a week by artificial vagina method. Semen samples were diluted with Tris egg yolk citrate extender and Black cumin (*Nigella sativa*) seeds aqueous extract was supplemented at different concentrations *viz.* 0% (T_1), 3% (T_2), 4% (T_3), 5% (T_4) (pH 6.5-6.8) maintaining final concentration of 100×10^6 sperm/ml. Lipid Peroxidation (MDA) and Reduced Glutathione (GSH) were evaluated at just after dilution (Initial), Pre-freeze and Post thaw stage (24 hours after cryopreservation). Result showed that supplementation of 5% Black cumin (*Nigella sativa*) seeds aqueous extract lowers the Lipid peroxidation (MDA) level and enhances Reduced Glutathione (GSH) level in Pre-freeze and Post thaw stages as compared to control and other supplemented group, which indicate that it is an effective antioxidant which increase the sperm antioxidant defense in cryopreserved Surti buck semen.

Keywords: *Nigella sativa*, black cumin, egg yolk citrate, oxidative stress

Introduction
India's rural population depend on agriculture and dairy for economic growth and play important socio-economic roles. More than 60% of the population is engaged in agriculture and allied activities. About 27.8% of the total livestock population is contributed by goats as per 20th Livestock Census. In India, as per 20th livestock census, the nation produces the most goat milk in the world, accounting for 26.31% of global production. To improve production potential of goat, the need for genetic improvement *via* implementation of Artificial Insemination (AI) from superior sires through semen preservation is crucially required. In the practise of artificial insemination, it is critical to preserve the fertilizing capacity of fresh sperm for as long as possible. During the process of cryopreservation, endogenous antioxidant concentration, semen quality and fertility become compromised due to the generation of reactive oxygen species (ROS) (Holt, 2000) [1]. The primary antioxidant system in seminal plasma acts as defensive machine by the aid of some enzymes such as glutathione peroxidase, superoxide dismutase, and catalase, which scavenge reactive oxygen species (ROS) resulting from lipid peroxidation (Lopez-Fernandez *et al.*, 2007) [2]. Reactive oxygen species (ROS) like hydroxyl, superoxide, nitric oxide, and hydrogen peroxide (H_2O_2) interact with sperm plasma membrane resulting in the occurrence of lipid peroxidation (LPO). The antioxidant capacity of mammalian semen is not sufficient to prevent oxidative stress during freezing (Bucak *et al*., 2010) [3].Therefore, over a few decades, researchers had studied the potential of antioxidant supplementations to the semen extender to overcome this problem. One such natural antioxidant containing plant, Black cumin (*Nigella sativa*) belongs to family Ranunculaceae. Some chemical compositions of polyphenol antioxidants found in Black cumin (*Nigella sativa*) have potential to protect sperm membrane from lipid peroxidation (Maidin *et al*., 2018) [4]. Black cumin (*Nigella sativa*) seed extract is effective for improving the quality of sperm after vitrification (Nasiri *et al*., 2022) [5].

Nigella sativa has been found to be able to improve sperms quality of rabbit (Riad *et al*., 2004) [6], ram (Inanc *et al*., 2021) [7] and buffalo (Awan *et al*., 2018) [8] semen. Looking into the various properties of Black cumin (*Nigella sativa*) seeds extract, study has been undertaken to investigate the effect of Black cumin (*Nigella sativa*) seeds extract in tris egg yolk citrate extender on oxidative stress parameters of cryopreserved Surti buck semen.

Materials and Methods

Selection and Management of bucks

The study was conducted on four apparently healthy mature Surti bucks above one years of age maintained under the All India Coordinated Research Project (AICRP) on Surti Goat at Livestock Research Station, Kamdhenu University Navsari. The selected bucks were housed in a common covered pen and under naturally existing photoperiod and air temperature of the South Gujarat. The animals were allowed to graze between 2:30 PM to 4:30 PM and fed with good quality fodder ad libitum along with 500 gm of concentrate per animal per day. They were dewormed four times in a year using different types of dewormer and regularly vaccinated against common diseases *viz*. Peste des Petits Ruminants (PPR) and Foot and Mouth Disease (FMD). The selected bucks were housed in a common covered pen and separated from females. The bucks were trained to donate the semen in artificial vagina by using female (doe) as dummy. After completion of the training period of about one month, semen was collected regularly by using artificial vagina twice a week from each buck for up to 8 weeks and total 64 semen ejaculates (16 ejaculates from each buck) were collected.

Preparation of Black cumin (*Nigella sativa*) aqueous extract

The seeds were carefully washed with clean water to get rid of dust and dirt. They were air shade dried for four days at room temperature and pulverized into a fine powder using mixer grinder. 100 gm of dried seed powder was extracted with water in glass container covered with aluminum foil and allowed to stand at room temperature for a period of 24 hours with frequent agitation until soluble matter had dissolved. The aqueous extract of seeds of Black cumin (*Nigella sativa*) was prepared through liquid partition method. The aqueous extract was stored in vial and kept in a refrigerator at -20 °C prior to sample preparation for subsequent analyses.

Semen collection, experimental group and cryopreservation

Semen was collected from all the selected bucks at early morning between 6.30 AM to 7.30 AM with the help of Eight-inch Artificial Vagina (AV) maintaining inner temperature of 40 °C to 42 °C and sufficient pressure. In order to maintain quality of semen, all the parts of artificial vagina are properly sterilized and for each buck separate Artificial vagina was used and during collection buck apron was applied to prevent further contamination. In order to increase the semen volume as well as eliminate individual buck variability the ejaculates of all four bucks were pooled. Only semen samples with initial motility ≥ 70% were considered for further processing.

The pooled semen was extended with tris egg yolk citrate extender to achieve final concentration of 100×10^6 sperm/ml. The diluted semen was separated into four equal aliquots, and each aliquot was treated with different concentrations of Black cumin (*Nigella sativa*) seeds aqueous extract *viz.* 0% (control T_1), 3% (T_2), 4% (T_3) and 5% (T_4) (pH 6.5-6.8). The extended semen from all the groups were examined at just after dilution (Initial), pre-freeze and post thaw (24 hours after cryopreservation) stages for Lipid Peroxidation (MDA) and Reduced Glutathione (GSH). Lipid peroxidation of spermatozoa was measured by determining the concentration of Malondialdehyde (MDA) production based on Thiobarbituric acid reaction (TBA) as an indicator for lipid peroxidation according to the method described by Perumal *et al*. (2016) [9] and Banday *et al*. (2017) [10]. Reduced glutathione (GSH) was estimated as per method described by Sedlak and Lindsay (1968) [11].

Statistical analysis

For statistical analysis, Descriptive analysis was carried out and mean ± SE was calculated for all the antioxidant semen parameters. The test of significance for above parameters was made by analysis of variance (ANOVA) and the mean difference between the bucks were tested by using Duncan's new Multiple Range test (DNMRT) at 5 and 1 percent level of significance.

Results and Discussions

Lipid Peroxidation (Malondialdehyde)

The mean initial lipid peroxidation (MDA) levels (nmol/ml) was non-significantly differed between T_1 (5.06±0.10), T_2 (4.93±0.09), T_3 (4.83±0.09) and T_4 (4.85±0.10) groups (Table 1). Pre-freeze mean lipid peroxidation (MDA) level (nmol/ml) was significantly ($p<0.01$) lower in T_4 (5.62±0.08) group as compared to T_3 (5.94±0.11), T_2 (6.16±0.09) and T_1 (6.35±0.09) groups. Pre-freeze mean MDA level (nmol/ml) in T_2 (6.16±0.09) and T_3 (5.94±0.11) group was significantly lower ($p<0.01$) as compared to T_1 (6.35±0.09) groups and both the groups differed non-significantly with each other. Post-thaw mean lipid peroxidation (MDA) level (nmol/ml) was significantly ($p<0.01$) lower in T_4 (6.63±0.09) group as compared to T_3 (7.16±0.10), T_2 (7.50±0.09) and T_1 (8.08±0.09) groups. Post-thaw mean lipid peroxidation (MDA) (nmol/ml) level was significantly ($p<0.01$) lower in T_3 (7.16±0.10) group as compared to T_2 (7.50±0.09) and T_1 (8.08±0.09) groups while, post-thaw mean lipid peroxidation (MDA) level (nmol/ml) was significantly ($p<0.01$) lower in T_2 (7.50±0.09) group as compared to T_1 (8.08±0.09) group.

The corresponding overall mean lipid peroxidation (MDA) level (nmol/ml) irrespective of treatment groups were increased with increasing preservation time at initial (4.92±0.05), pre-freeze (6.02±0.06) and post-thaw (7.34±0.08) stage. The overall mean lipid peroxidation (MDA) level (nmol/ml) irrespective of different treatment groups significantly ($p<0.01$) differed at various stages of cryopreservation. Moreover, in T_1, T_2, T_3, T_4 groups mean lipid peroxidation (MDA) level (nmol/ml) was significantly ($p<0.01$) lower at initial (5.06±0.10, 4.93±0.09, 4.83±0.09 and 4.85±0.10) stage as compared to pre-freeze (6.35±0.09, 6.16±0.09, 5.94±0.11 and 5.62±0.08) and post-thaw (8.08±0.09, 7.50±0.09, 7.16±0.10 and 6.63±0.09) stage. Furthermore, mean lipid peroxidation (MDA) level (nmol/ml) among initial, pre-freeze and post-thaw stage differed significantly ($p<0.01$) in all the groups.

Table 1: Antioxidant effect of different concentrations of Black cumin (*Nigella sativa*) seeds aqueous extract on Lipid Peroxidation (MDA) level of Surti buck semen at various stages of cryopreservation (Mean±SE)

Groups	Lipid Peroxidation (MDA) (nmol/ml) (n=16)			Overall (n= 48)	F-value	P-value
	Initial	Pre-freeze	Post-thaw			
T_1	$5.06\pm0.10^{a}{}_{z}$	$6.35\pm0.09^{a}{}_{y}$	$8.08\pm0.09^{a}{}_{x}$	6.50 ± 0.19^{a}	264.45**	0.00
T_2	$4.93\pm0.09^{a}{}_{z}$	$6.16\pm0.09^{ab}{}_{y}$	$7.50\pm0.09^{b}{}_{x}$	6.20 ± 0.16^{ab}	206.11**	0.00
T_3	$4.83\pm0.09^{a}{}_{z}$	$5.94\pm0.11^{b}{}_{y}$	$7.16\pm0.10^{c}{}_{x}$	5.98 ± 0.15^{bc}	129.43**	0.00
T_4	$4.85\pm0.10^{a}{}_{z}$	$5.62\pm0.08^{c}{}_{y}$	$6.63\pm0.09^{d}{}_{x}$	5.70 ± 0.12^{c}	90.83**	0.00
Overall (n=64)	$4.92\pm0.05_{z}$	$6.02\pm0.06_{y}$	$7.34\pm0.08_{x}$	--	4.66**	0.00
F-value	1.21	11.35**	41.02**	365.20**	--	--
P-value	0.31	0.00	0.00	0.00	--	--

[a-c] Means with different superscript within a column (between the groups) differs significantly at $p<0.01$. [x-z] Means with different subscript between a column (between various stages) differs significantly at $p<0.01$. **$p<0.01$

T_1 – control, T_2 – 3% Black cumin (*Nigella sativa*) seeds aqueous extract, T_3 - 4% Black cumin (*Nigella sativa*) seeds aqueous extract, T_4 - 5% Black cumin (*Nigella sativa*) seeds aqueous extract.

The corresponding overall mean lipid peroxidation (MDA) level (nmol/ml) irrespective of time interval was significantly ($p<0.01$) lower in T_4 (5.70±0.12) group as compared to T_2 (6.20±0.16) and T_1 (6.50±0.19) groups. Overall mean MDA level (nmol/ml) in T_3 (5.98±0.15) group was non-significantly lower as compared to T_2 (6.20±0.16) and significantly lower as compared to T_1 (6.50±0.19) group. While Overall mean MDA level (nmol/ml) in T_2 (6.20±0.16) group was non-significantly lower as compared to T_1 (6.50±0.19) group.

Lowest mean lipid peroxidation (MDA) level (nmol/ml) was found at initial, pre-freeze and post-thaw stage in T_4 group (4.85±0.10, 5.62±0.08 and 6.63±0.09) followed by T_3 (4.83±0.09, 5.94±0.11 and 7.16±0.10) and T_2 (4.93±0.09, 6.16±0.09 and 7.50±0.09) group, while highest mean MDA level (nmol/ml) was found in T_1 group at initial (5.06±0.10), pre-freeze (6.35±0.09) and post-thaw (8.08±0.09) stage.

In the present study, significantly ($p<0.01$) lower MDA levels (nmol/ml) in Surti buck semen was observed in 5% *Nigella sativa* aqueous extract supplemented group at pre-freeze and post-thawed stage as compared to 4% *Nigella sativa* aqueous extract and control group. In accordance to the present findings Mehdipour *et al.* (2016) [12]; Banday *et al.* (2017) [10]; and El-Seadawy *et al.* (2022) [13] in ram and Seifi-Jamadi *et al.* (2017) [14] in goat buck also reported lower MDA levels in *Camellia sinensis;* taurine; moringa leaf and quercetin extract supplemented in extender group as compared to control group, respectively. Moreover, El-Gindy (2022) [15] and El-Tohamy *et al.* (2010) [16] reported oral administration of *Nigella sativa* extract in different quantity showed lower MDA levels (nmol/ml) in rabbit buck semen as compared to control group.

Similarly, Mehdipour *et al.* (2016) [12] reported significantly ($p<0.05$) lowest MDA (nmol/ml) level in 10 mg/l green tea extract (1.4 ± 0.2) supplemented in extender group followed by in 5 mg/l green tea extract (2.4 ± 0.2) group as compared to control group (3.1 ± 0.2). LPO may stimulate phenomenon similar to apoptotic result in decreased life span in cryopreserved spermatozoa. 10 mg/l of green tea extract could inhibit expression of proapoptotic genes resulting in decreased sperm cell death which lower MDA levels. In accordance to present findings, Seifi-Jamadi *et al.* (2017) [14] found significantly ($p<0.001$) lower MDA (nmol/ml) in quercetin 10 µM (5.81 ± 0.44) group as compared to 20 µM (6.78 ±0.17) and control (6.96±0.22) group. Banday *et al.* (2017) [10] also found significantly ($p<0.05$) lower seminal MDA level (nmol/ml) in 40 mM taurine supplemented in tris-based extender group (3.26 ± 0.21) as compared to control (4.43 ± 0.62) group.

El-Seadawy *et al.* (2022) [13] reported that addition of moringa leaf methanolic extract in cryopreservation media at 0.48 mg/ml and 0.64 mg/ml showed significantly ($p<0.05$) lower post thaw MDA levels as compared to control group in ram semen. They concluded that phenolic compounds of moringa leaves are strong electron donors and may be able to stop the radical chain reaction by converting free radical to stable product (Lobo *et al.* 2010) [17]. Contrary to present findings, Inanc *et al.* (2021) [7] reported addition of thymoquinone in tris-based extender at 25 µg/mL showed higher post thaw MDA levels as compared to control group in ram semen. They concluded that other studies showed administration of thymoquinone and *Nigella sativa* on feeding, injection etc could be the reason while addition of thymoquinone in extender could not produce significant change in lipid peroxidation profile in extender. Thymoquinone one of the major content of *Nigella sativa* has an antioxidant protective effect that may repress the expression of cyclooxygenase-2 enzyme and lipid peroxidation (Al Wafai, 2013) [18].

Reduced glutathione: The mean initial reduced glutathione (GSH) (nmol/ml) levels differed non-significantly among T_1 (5.71±0.12), T_2 (5.80±0.11), T_3 (5.84±0.11) and T_4 (5.87±0.11) groups (Table 2). Pre-freeze mean GSH (nmol/ml) level was significantly ($p<0.01$) higher in T_4 (5.42±0.10) group as compared to T_3 (5.07±0.12), T_2 (4.84±0.12) and T_1 (4.54±0.13) groups. Pree-freeze mean GSH (nmol/ml) level in T_3 (5.07±0.12) group was significantly ($p<0.01$) higher as compared to T_1 (4.54±0.13) group and non-significantly higher as compared to T_2 (4.84±0.12) group. Pre-freeze mean GSH (nmol/ml) level in T_2 (4.84±0.12) group was non-significantly higher as compared to T_1 (4.54±0.13) group. Post-thaw mean GSH (nmol/ml) level was significantly ($p<0.01$) higher in T_4 (4.44±0.11) group as compared to T_3 (4.11±0.12), T_2 (3.77±0.11) and T_1 (3.31±0.09) groups. Post-thaw mean GSH (nmol/ml) level in T_3 (4.11±0.12) group was significantly ($p<0.01$) higher as compared to T_2 (3.77±0.11) and T_1 (3.31±0.09) groups. While post-thaw mean GSH (nmol/ml) level in T_2 (3.77±0.11) group was significantly ($p<0.01$) higher as compared to T_1 (3.31±0.09) group.

The corresponding overall mean GSH (nmol/ml) level irrespective of treatment groups were decreased with increasing preservation time at initial (5.80±0.06), pre-freeze (4.97±0.07) and post-thaw (3.91±0.08) stage. The overall mean GSH (nmol/ml) level irrespective of different treatment groups differed significantly ($p<0.01$) among various stages of cryopreservation.

Moreover, in T_1, T_2, T_3, T_4 groups mean GSH (nmol/ml) level was significantly ($p<0.01$) higher at initial (5.71±0.12, 5.80±0.11, 5.84±0.11 and 5.87±0.11) stage as compared to pre-freeze (4.54±0.13, 4.84±0.12, 5.07±0.12 and 5.42±0.10) and post-thaw stage (3.31±0.09, 3.77±0.11, 4.11±0.12 and 4.44±0.11) stage.

Table 2: Antioxidant effect of different concentrations of Black cumin (*Nigella sativa*) seeds aqueous extract on Reduced Glutathione (GSH) level of Surti buck semen at various stages of cryopreservation (Mean±SE)

Groups	Reduced Glutathione (GSH) (nmol/ml) (n=16)			Overall (n= 48)	f-value	p-value
	Initial	Pre-freeze	Post-thaw			
T_1	$5.71±0.12^{a}_{x}$	$4.54±0.13^{c}_{y}$	$3.31±0.09^{d}_{z}$	$4.52±0.16^{c}$	109.46**	0.00
T_2	$5.80±0.11^{a}_{x}$	$4.84±0.12^{bc}_{y}$	$3.77±0.11^{c}_{z}$	$4.80±0.14^{bc}$	77.96**	0.00
T_3	$5.84±0.11^{a}_{x}$	$5.07±0.12^{b}_{y}$	$4.11±0.12^{b}_{z}$	$5.01±0.12^{ab}$	55.39**	0.00
T_4	$5.87±0.11^{a}_{x}$	$5.42±0.10^{a}_{y}$	$4.44±0.11^{a}_{z}$	$5.24±0.11^{a}$	44.67**	0.00
Overall (n=64)	$5.80±0.06_{x}$	$4.97±0.07_{y}$	$3.91±0.08_{z}$	--	5.38**	0.00
F-value	0.337	9.749**	19.730**	197.128**	--	--
P-value	0.789	0.00	0.00	0.00	--	--

[a-d] Means with different superscript within a column (between the groups) differs significantly at $p<0.01$. [x-z] Means with different subscript between a column (between various stages) differs significantly at $p<0.01$. **$p<0.01$

T_1 – control, T_2 – 3% Black cumin(*Nigella sativa*)seeds aqueous extract, T_3 - 4% Black cumin(*Nigella sativa*) seeds aqueous extract, T_4 - 5% Black cumin(*Nigella sativa*)seeds aqueous extract.

The corresponding overall mean GSH (nmol/ml) level irrespective of time interval was significantly ($p<0.01$) higher in T_4 (5.24±0.11) group as compared to T_2 (4.80±0.14) and T_1 (4.52±0.16) and non-significantly higher as compared to T_3 (5.01±0.12) groups. Overall mean GSH (nmol/ml) level in T_3 (5.01±0.12) group was significantly ($p<0.01$) higher as compared to T_1 (4.52±0.16) group and non-significantly higher as compared to T_2 (4.80±0.14) group. While overall mean GSH (nmol/ml) level in T_2 (4.80±0.14) group was significantly ($p<0.01$) higher as compared to T_1 (4.52±0.16) group. Highest mean GSH (nmol/ml) level was found at initial, pre-freeze and post-thaw stage in T_4 (5.87±0.11, 5.42±0.10 and4.44±0.11) group followed by T_3 (5.84±0.11, 5.07±0.12 and 4.11±0.12) and T_2 (5.80±0.11, 4.84±0.12 and 3.77±0.11) group. While lowest mean GSH (nmol/ml) level was found in T_1 group at initial (5.71±0.12), pre-freeze (4.54±0.13) and post-thaw (3.31±0.09) stage.

Result from the present study showed that GSH (nmol/ml) level was increasing in dose dependent manner in various stages of preservation of semen. In present study GSH (nmol/ml) level was significantly ($p<0.01$) higher in 5% Black cumin (*Nigella sativa*) seeds aqueous extract in pre-freeze and post thaw stages followed by 4% Black cumin (*Nigella sativa*) seeds aqueous extract group as compared to control group. In accordance to present findings, Inanc *et al.* (2021) [7] in ram, Perumal *et al.* (2013) [19] in bull, Sariozkan *et al.* (2009) [20] in bull, Atessahin *et al*. (2008) [21] in goat buck and Bucak *et al.* (2007) [22] in ram reported higher GSH (nmol/ml) levels in higher concentrations of various antioxidant added in extender group as compared to other groups. In present study, higher post-thawed semen GSH (nmol/ml) level in 5% Black cumin (*Nigella sativa*) seeds aqueous extract group was in agreement with Inanc *et al.* (2021) [7], who reported that addition of thymoquinone at 100 µg/mL and 50 µg/mL in extender showed non-significantly increased GSH (nmol/ml) level as compared to control group. Similarly, Atessahin *et al*. (2008) [21] reported higher frozen thawed semen GSH (nmol/ml) level in 15 mM cysteine concentration (3.45 ± 0.28) followed by 10 mM cysteine (3.27 ± 0.11) as compared to control group (2.50 ± 0.08).

Results from this study showed dose-dependent increase in GSH levels had accordance with results of the present study as increasing concentrations of *Nigella sativa* extract gave higher levels of GSH. Perumal *et al.* (2013) [19] reported addition of different concentration of taurine could increase GSH levels. They found higher GSH levels in dose dependent manner in 25 mM and 50 mM in bull semen. However, counter results was reported by Sariozkhan *et al.* (2009) [20] in frozen thawed bull semen. They reported addition of taurine at 2 mM and cysteine at 2 mM in Bioxcell commercial extender could not elevate GSH levels as compared to control group. Phytochemical analysis indicated the rich presence of unsaturated fatty acids (Linoleic acids 55.6%, Stearic acids 3.4%, Oleic acid 23.4%, Palmitic acid 12.5%) in *Nigella sativa* seeds (Nickwar *et al.* 2003) [23] that play important role in antioxidative activity. Beyond that antioxidant property of *Nigella sativa* is due to thymoquinone, thymol and di-thymoquinone and minerals like iron, sodium, potassium, and copper (Nasiri *et al.* 2022) [24] which act as co-factors for different enzymes of antioxidant system contributing the antioxidant properties. The changes in preservation protocols, extender formulations, additive concentrations and animal species may explain different behaviours of antioxidant capacities (Bucak *et al.*, 2007) [22].

Conclusion

Lipid peroxidation (MDA) level was observed to be decreasing with increase in Black cumin (*Nigella sativa*) seeds aqueous extract concentration at pre freeze and post thaw stages with lowest level in 5% Black cumin (*Nigella sativa*) seeds aqueous extract supplementation in tris egg yolk citrate extender for cryopreservation of Surti buck semen. Reduced Glutathione (GSH) values were observed to be increasing with increase in Black cumin (*Nigella sativa*) seeds aqueous extract concentration at initial, pre freeze and post thaw stages with highest GSH activity was observed in 5% Black cumin (*Nigella sativa*) seeds aqueous extract in tris egg yolk citrate extender group which indicate that it is an effective antioxidant which increase the sperm antioxidant defense in cryopreserved Surti buck semen.

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