Survival Rate and Selected Essential Metals in *Drosophila Melanogaster* Fed with Different Concentrations Of Chitosan

***[1]Igharo, O.G., [1]Omonye, I.P., [2]Osakue, J.O. and [1]Osadolor, H.B.**
[1]Department of Medical Laboratory Sciences, School of Basic Medical Sciences,
College of Medical Sciences, University of Benin, Benin City, Nigeria
[2]Department of Physiology, School of Basic Medical Sciences,
College of Medical Sciences, University of Benin, Benin City, Nigeria

***Corresponding author:**
E-mail: osaretin.igharo@uniben.edu
Tel.: 08038664896

Abstract

Background: Chitosan is a copolymer of glucosamine and N-acetylglucosamine and it has an amine functional group which is strongly reactive with metal ions. **Objective:** To evaluate the effect of dietary inclusion of varied concentrations of chitosan on survival rate and selected essential metal levels in Harwich Strain of *Drosophila melanogaster*. **Materials and Methods:** For the survival study, the flies were allowed to feed on different concentrations of chitosan meal for 14 days. The survival assay was noted by taking daily readings of the number of death in each treatment vial, and standard Survival Assay was used to estimate their survival rate. For the chitosan treatment, seven treatment groups (I-VII) were fed with 10µg, 20µg, 40µg, 320µg, 640µg, 1mg chitosan meal respectively. Group VII was the control containing no chitosan treatment. Each treatment vials had 50 *Drosophila melanogaster* flies and they were allowed to feed on the meal for seven (7) days prior to biochemical assay. The flies were homogenized and levels of the essential metals (copper, zinc and selenium) were determined using the inductively coupled plasma –mass spectrometry. **Results:** Flies fed with varying concentrations of chitosan in diet had higher survival rate than the control group. Levels of copper, zinc, and selenium in the control group were not significantly different compared with all groups of flies fed with the varied chitosan concentrations in meal. **Conclusion:** It could be deduced that dietary inclusion of chitosan in Drosophila meals may prolong fly survival and but may not modulate the levels of the essential metals.

Keywords: *Drosophila Melanogaster*, Chitosan, Survival rate, Essential metals

Introduction

The term chitosan refers to chitin that has been partially deacetylated. Chitin on the other hand is obtained mostly from exoskeletons of insects, crustaceans such as shrimps and crabs and even cell walls of fungi. It is the most abundant polysaccharide after cellulose. Chitosan is composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine (1). Its high nitrogen atom contents and hydroxyl group allows its uptake of metal ions through the process of chelation and ion exchange (2). Chitosan is cheap, biodegradable and nontoxic to mammals. Its wide applications, such as drug delivery, metal ion removal, dye removal, agricultural uses, in cosmetics, water engineering, food processing etc., has led to the increase in its routine applications.

Trace elements or inorganic micronutrients are chemical elements required by living organisms in minute amounts (that is less than 0.1 percent by volume [1,000 parts per million]), and usually parts of vital enzyme systems in the body. Although these elements account for only 0.02% of the total body weight, they play significant roles, e.g. as active centers of enzymes or as trace

bioactive substances (3, 4). A major outcome of trace element deficiencies is reduced activity of the concerned enzymes (5).

The fruit fly, *Drosophila melanogaster* has been widely used as a model organism to understand many molecular and biological processes in human (6, 7). The use of *Drosophila melanogaster* eliminates the problem of obtaining ethical clearance as applicable in the use of laboratory animals like mouse, rats or rabbits. It also has many other advantages such as its size being 3-4mm long, making it possible for smaller amounts of assay reagents, very importantly, an estimated 75% of the human genes implicated in diseases have homologs in Drosophila, with about 90% nucleotide sequence identified in some of its species (8). Flies can easily be handled, bred (having a short life span) and genetically manipulated in large numbers (9). There are accumulating reports on the use of *Drosophila* to study potential toxic effects of different toxicants, including metal compounds (6,7,10).

Awareness and human application of chitosan is on the increase. It has been applied in hair care product formulation, as flavouring and colouring agent in baked food, as metal trapping agent, in tissue engineering, wound healing and dressing material, tumor cell growth inhibition agent, as an antimicrobial agent (11).

With its varied known and prospective applications, the need for extensive studies on its possible toxicity in different experimental models cannot be overemphasized. The present study therefore investigates the effect of dietary inclusion of different concentration of chitosan on survival and selected essential metal levels in *Drosophila melanogaster.*

Materials and Methods

Drosophila melanogaster stock culture

D. melanogaster (Harwich strain) from National Species Stock Center (Bowling Green, OH, USA), was obtained from Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria. The flies were maintained and reared on normal diet made up of corn meal medium containing 1% w/v brewer's yeast and 0.08% v/w Nipagin at room temperature under 12-hr dark/light cycle conditions in the Department of physiology, University of Benin, Nigeria. All the experiments were carried out with the same *D. melanogaster* strain.

Chitosan extraction

Chitosan was extracted from chitin-rich snail shells by the processes of deproteinization, demineralization and deacetylation (1).

Deproteinization- powdered shell was boiled with 4% weight/volume (w/v) NaOH for 2hours.

Demineralization- the sample was demineralized using 1% HCl with four times its quantity. The samples were allowed to soak for 24 hours to remove the minerals (mainly calcium carbonate).

Deacetylation was carried out by adding 50% NaOH and boiled at 100°C for 2 hours in a water bath (12). The extracted Chitosan was further subjected to treatment to achieve a dry whitish powder chitosan. In summary, the processing of chitosan from the crustacean shells was based on the protocol described by Randy *et al* (1).

Experimental design

The flies (both male and female, 3–5 days old) were divided into seven groups containing 50 flies each. Group I (Control) was placed on normal diet alone, while groups II–VII, based on discretion, were placed on basal diet containing; 10µg, 20µg, 40µg, 320µg, 640µg and 1mg of chitosan in the diet respectively, as shown below.

Group I: Control (basal diet)
Group II: Basal diet + 10 µg chitosan
Group III: Basal diet + 20 µg chitosan
Group IV: Basal diet + 40 µg chitosan
Group V: Basal diet + 320 µg chitosan
Group VI: Basal diet + 640 µg chitosan
Group VII: Basal diet +1 mg chitosan

The flies were exposed to these treatments for 7 and 14 days, and the vials containing flies were maintained at room temperature. Each experimental group was carried out in five independent vials.

Survival study

Survival study was conducted to assess the effect

of dietary inclusion of chitosan on survival rate of flies after 14 days of exposure. Flies (both gender, 3–5 days old) were divided into seven treatment groups, with each group having 5 vials, containing 50 flies each. Each group was exposed to the different chitosan concentrations in meal (10ug, 20ug, 40ug, 320ug, 640ug and 1mg). The flies were observed daily for the incidence of mortality, and the survival was determined by counting the number of dead flies. The survival rate was determined with all the concentrations, and both the living and dead flies were recorded daily. At the end of the chosen duration for the survival rate experiment (14 days), the data obtained were accumulated and plotted as percentage of living and dead flies. The results obtained were compared with that of the control following previously reported protocols (13,14).

Tissue homogenate preparation for bioassay of copper, zinc and selenium

For the determination of biochemical assays, a second experiment with similar experimental design was set up. Flies (1 to 3 days old, male and female) were divided into seven groups, with each group having 5 vials containing 50 flies per vial. Each group had five vials containing 50 flies (including males and females). Group I (Control) had normal saline in diet while groups II - VII had the varying concentrations (described above) of chitosan in diet for 7 days. At the end of the treatment period, flies were anaesthetized using ice, weighed, and homogenized in cold 0.1M phosphate buffer, pH 7.0 (1:10 w/v). The resulting homogenates were centrifuged at 10,000 × g, at 4°C for 10 minutes in a Kenxin refrigerated centrifuge Model KX3400C (KENXIN Intl. Co., Hong Kong). Subsequently, the supernatants were separated from the pellets into labelled Eppendorf tubes, and used for the bioassay of copper, zinc and selenium. The trace metal analysis was carried out at the analytical services laboratory of the International Institute for Tropical Agriculture, IITA, Ibadan, using the inductively coupled plasma mass spectrometer (ICP-MS) (Thermo Elemental, X series I, Germany), based on methods described by Fong *et al*., (15).

Statistical Analysis

The data were analyzed using the statistical package for social sciences program (SPSS) version 21.0 (Chicago IL) and presented as mean ± standard deviation. Precisely, the One Way Analysis of Variance (ANOVA) and Turkey's Post Hoc test were used at $\alpha 0.05$.

Results

Figure 1 is the Survival Curve indicating the effect of the various concentrations of chitosan in diet on the fly survival. As indicated, the fly survival rate (23 %) in group V (with 320 µg chitosan) was highest compared with control and other treatment other groups. Additionally, treatment groups with 10µg, 20µg, 40µg, 640µg, and 1mg chitosan in meal showed higher survival rates of 20%, 22%, 15%, 10%, and 18% respectively at day 14 when compared to the control group.

Levels of all three trace metals in Control group were significantly higher compared with values obtained in the treatment groups (Table 1); while among the treatment groups, the trace metals- zinc, copper and selenium did not vary significantly (Tables 2 -7).

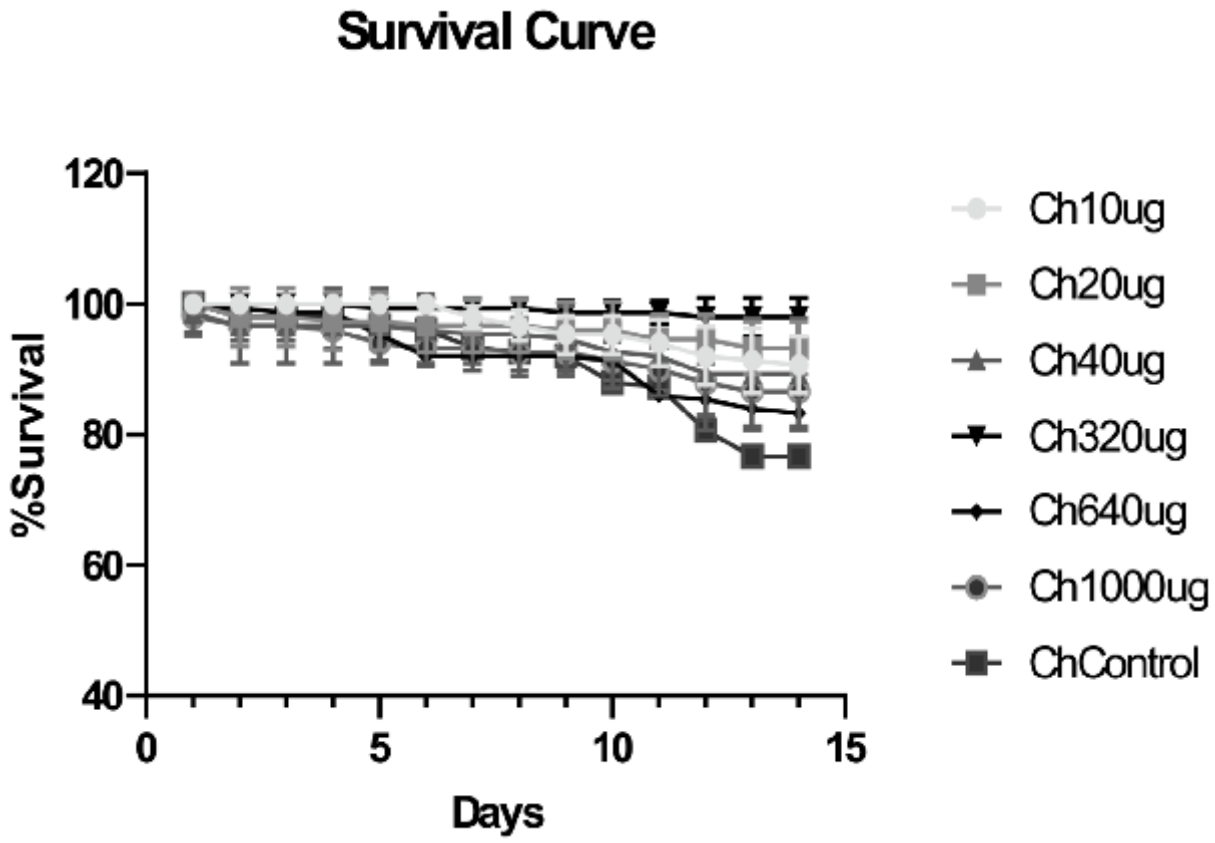

Fig. 1 Survival curve indicating the effect of the various concentrations of chitosan in diet on fly survival.

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Table 1 Copper, Zinc and Selenium levels in Control and Treatment Groups

Parameters	Control N = 5	10ug of Chitosan meal N =5	20ug of chitosan meal N = 5	40ug of chitosan meal N = 5	320ug of chitosan meal N = 5	640ug of chitosan meal N= 5	1mg of chitosan meal N = 5	F valves	P valves
Copper (µg/dL)	137.50+4.75	82.81+13.42	95.55+9.19	96.35+14.98	96.55+14.89	104.78+12.27	93.91+11.79	7.865	PC<0.001 P1<0.001 P2<0.001 P3<0.001 P4=0.001 P5= 0.008 P6<0.001
Zinc (µg/dL)	115.17±4.75	60.48±13.42	73.22±9.19	74.01±14.98	74.21±14.89	82.45±12.27	71.58±11.79	7.870	PC< 0.001 P1<0.001 P2<0.001 P3<0.001 P4=0.001 P5=0.008 P6<0 001
Selenium (µg/dL)	69.46±1.70	49.93±4.79	54.48±3.28	54.76±5.95	54.83±5.31	57.78±4.38	53.89±4.21	7.865	PC< 0.001 P1<0.001 P2<0.001 P3=0.001 P4=0.001 P5=0.008 P6<0.001

Keys:
N=Number of treatment vial in each group
PC = P value of combined comparison
P1 = P value of control vs. 10 µg chitosan meal
P2 = P value of control vs. 20 µg chitosan meal
P3 = P value of control vs. 40 µg chitosan meal
P4 = P value of control vs. 320 µg chitosan meal
P5 = P value of control vs. 640 µg chitosan meal
P6 = P value of control vs. 1mg chitosan meal

Table 2 Comparison of Zinc, Copper and Selenium in 10µg chitosan meal group with other treatment groups

Parameters	Concentration	P valve	Level of significance
Zinc (µg/dL)	20µg	0.780	Not significant
	40µg	0.730	Not significant
	320µg	0.717	Not significant
	640µg	0.213	Not significant
	1mg	0.868	Not significant
Copper (µg/dL)	20µg	0.780	Not significant
	40µg	0.730	Not significant
	320µg	0.717	Not significant
	640µg	0.213	Not significant
	1mg	0.868	Not significant
Selenium (µg/dL)	20µg	0.780	Not significant
	40µg	0.730	Not significant
	320µg	0,717	Not significant
	640µg	0.213	Not significant
	1mg	0.868	Not significant

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Table 3 Comparison of Zinc, Copper and Selenium in 20µg chitosan meal group with other treatment groups

Parameters	Concentration	P valve	Level of significant
Zinc (µg/dL)	10µg	0.780	Not significant
	40µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.888	Not significant
	1mg	1.000	Not significant
Copper (µg/dL)	10µg	0.780	Not significant
	40µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.888	Not significant
	1mg	1.000	Not significant
Selenium (µg/dL)	10µg	0.780	Not significant
	40µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.888	Not significant
	1mg	1.000	Not significant

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Table 4 Comparison of Zinc, Copper and Selenium in 40µg chitosan meal group with other treatment groups

Parameters	Concentration	P valve	Level of significant
Zinc (µg/dL)	10µg	0.730	Not significant
	20µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.888	Not significant
	1mg	1.000	Not significant
Copper (µg/dL)	10µg	0.730	Not significant
	20µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.888	Not significant
	1mg	1.000	Not significant
Selenium (µg/dL)	10µg	0.730	Not significant
	20µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.888	Not significant
	1mg	1.000	Not significant

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Table 5 Comparison of Zinc, Copper and Selenium in 320µg chitosan meal group with other treatment groups

Parameters	Concentration	P valve	Level of significant
Zinc (µg/dL)	10µg	0.717	Not significant
	20µg	1.000	Not significant
	40µg	1.000	Not significant
	640µg	0.931	Not significant
	1mg	1.000	Not significant
Copper (µg/dL)	10µg	0.929	Not significant
	20µg	1.000	Not significant
	40µg	1.000	Not significant
	640µg	0.997	Not significant
	1mg	1.000	Not significant
Selenium (µg/dL)	10µg	0.868	Not significant
	20µg	1.000	Not significant
	40µg	1.000	Not significant
	640µg	0.931	Not significant
	1mg	1.000	Not significant

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Table 6 Comparison of Zinc, Copper and Selenium in 640µg chitosan meal group with other treatment groups

Parameters	Concentration	P valve	Level of significant
Zinc (µg/dL)	10µg	0.213	Not significant
	20µg	0.888	Not significant
	40µg	0.924	Not significant
	320µg	0.931	Not significant
	1mg	0.791	Not significant
Copper (µg/dL)	10µg	0.213	Not significant
	20µg	0.888	Not significant
	40µg	O.924	Not significant
	320µg	0.931	Not significant
	1mg	0.791	Not significant
Selenium µg/dL)	10µg	0.213	Not significant
	20µg	0.888	Not significant
	40µg	O.924	Not significant
	320µg	0.931	Not significant
	1mg	0.791	Not significant

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Table 7 Comparison of Zinc, Copper and Selenium in 1mg chitosan meal group with other treatment groups

Parameters	Concentration	P valve	Level of significant
Zinc (µg/dL)	10µg	0.868	Not significant
	20µg	1.000	Not significant
	40µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.791	Not significant
Copper (µg/dL)	10µg	0.868	Not significant
	20µg	1.000	Not significant
	40µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.791	Not significant
Selenium (µg/dL)	10µg	0.868	Not significant
	20µg	1.000	Not significant
	40µg	1.000	Not significant
	320µg	1.000	Not significant
	640µg	0.791	Not significant

Keys:
Ch10ug = 10µg of Chitosan meal
Ch20ug = 20µg of chitosan meal
Ch40ug = 40µg of chitosan meal
Ch320ug = 320µg of chitosan meal
Ch640ug = 640µg of chitosan meal
Ch1mg = 1mg of chitosan meal
ChControl = Control

Discussion

Drosophila melanogaster is one of the alternative invertebrate models useful in toxicological testing. Drosophila has gained appreciation as a useful model for studying human diseases, including those caused by mutations in pathways controlling cellular metal homeostasis (9). It meets the standard of the European Centre for the Validation of Alternative Methods (ECVAM), Reduction, Refinement and Replacement (3RS) of the usage of laboratory animals (16).Chitosan has been widely used for different biological and biomedical applications recently due to its unique properties. For instance, it can be used in water treatment (17), wound-healing materials (18), pharmaceutical excipient or drug carrier (19), obesity treatment (20) and as a scaffold for tissue engineering (21). There is increased interest in pharmaceutical as well as biomedical applications of chitosan and its derivatives and significant development has been achieved.

In this study, incorporation of varying concentrations of chitosan into diet was used to determine the survival rate and the trace metal modulatory role of chitosan in *Drosophila melanogaster*. Studies have shown that Chitosan is effective for the removal of copper II, zinc II, and selenium in solution (22). In a related study, the high sorption capacities of modified chitosan for metal ions are of great use for the recovery of valuable metals or the treatment of contaminated effluents. A great number of chitosan derivatives have been obtained with the aim of adsorbing metal ions by including new functional groups

on the chitosan backbone. The new functional groups are incorporated into chitosan to increase the density of sorption sites, to change pH range for metal sorption and to change the sorption sites in order to increase sorption selectivity for the target (23)

The observed higher survival chance of the flies in the chitosan treated groups could possibly indicate that the flies were tolerant to the concentrations of chitosan used for the treatment, particularly the 320µg which appear to have the most suitable effect on the fly survival. The implication is that the 320µg chitosan meal could be the concentration the flies have a better adaption to. This enhanced survival may also point to the safety of chitosan in health applications such as its documented use as pharmaceutical excipient (1, 15).

Furthermore, the trace metals adsorptive properties of chitosan has been reported by Raman *et al*., (24) and its other metal-related interactions have also been studied, (11). Our observation of lower levels of zinc, copper and selenium associated with the chitosan-treated groups could be associated with the metal ion trapping properties of chitosan as previously documented (1,2,11).

Deficiencies of copper can result in hernias, aneurysms, blood vessel breakage manifesting as bruising or nose bleeds, iron deficiency anemia, osteoporosis and joint problems, brain disturbances, abnormalities in glucose and cholesterol metabolism (25). Additionally, increased susceptibility to infections due to poor immune function (neutropenia) (26), loss of pigment, weakness, fatigue, skin sores, poor thyroid function, irregular heart beat and low body temperature in man are also documented as conditions associated with copper deficiency. The possibility of excess or unregulated intake or exposure to cooper is well-known. Excessive copper intake can cause nausea, vomiting, abdominal pain and cramps, headache, dizziness, weakness, diarrhea, and a metallic taste in the mouth (associated with water containing copper concentrations greater than 6 mg/L) (25,26).

Excess zinc results in abdominal symptoms such as nausea, vomiting, loss of appetite, abdominal cramps. It also has effects on the central nervous system (CNS), producing symptoms such as headache (5, 25). Early aging, poor wound healing and gastroenteritis are some of the effect of zinc deficiency in the body (27).

Deficiency of selenium can lead to keshan and keshin Beck diseases. Manifestations of keshan and keshin Beck disease include necrosis of the myocardium, and atrophy, degeneration and necrosis of cartilage tissue respectively. In the same vein, selenium toxicity is characterized clinically by nausea, vomiting, nail discolouration, hair loss, fatigue and halitosis (28).

In the present study, the possible effects of excess amount of the selected essential metals may have counteracted or mitigated by the metal-modulatory roles of chitosan in the diets administered to the flies used in the present study. The fact that the levels of the three trace metals were similar in all the chitosan treated groups but lower than the control group, may indicate that dietary inclusion of chitosan could play a modulatory role in trace metal metabolism in *Drosophila melanogaster*.

Conclusion

Dietary inclusion of chitosan in *Drosophila* meals may enhance survival rate and modulate the levels of the essential metals, particularly copper, zinc and selenium in the fly.

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