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Effect of Dietary Inclusion of Chitosan on Survival and Selected Cellular Antioxidants in *Drosophila melanogaster*

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ABSTRACT: This study evaluated the effect of dietary inclusions of different concentrations of chitosan on survival rate and some oxidative stress markers in *Drosophila melanogaster* (Harwich strain). Flies were raised on diet supplemented with chitosan for 7 days and 14 days for survival assay. The survival assay was used to evaluate their survival rate. The flies were subsequently homogenized and the oxidative stress markers gluthathione, gluthathione-S-transferase and catalase were assayed. As observed, *Drosophila* flies fed with 10µg, 20µg, 40µg, 320µg, 640µg, 1mg chitosan meal had a higher survival rate compared to control. 320µg of chitosan meal had the highest survival rate (96%), indicating that the *Drosophila* flies was more tolerable to this concentration. It was observed that inclusions of chitosan in their meal especially at 320µg concentration increased their survival rate compared to control. Activities of gluthathione, catalase were increased in flies fed with diet supplemented with chitosan than in control. These results have shown that dietary inclusions of chitosan in various concentrations increased the survival rate of the flies than in control which contained only water and also increase some antioxidant enzymes in the flies than in control. Reduced antioxidant levels have been linked to accelerated aging and conditions leading to death like cancer. Based on the fact that antioxidant slow aging process and reduces risks of life threatening conditions. The result indicated that chitosan inclusion in *Drosophila* meals may modulate oxidative stress status in *Drosophila melanogaster* and increase survival rate.

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A large body of evidence have demonstrated that reactive oxygen species (ROS) including superoxide radicals ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) are the byproduct of metabolic biological systems, and processes like protein phosphorylation, activation of several transcriptional factors, apoptosis, immunity, and differentiation relies on the production of some ROS (Pizzano *et al.* 2017; Navarro *et al.* 2014; Rajendran *et al.* 2014). Going forward, a prelude to the mechanism underlying the production of ROS would be instructive. Production of ROS depend on enzymatic and nonenzymatic reactions. In respect of enzymatic reaction, ROS are produced via respiratory chain reaction, phagocytosis, prostaglandin synthesis and cytochrome P450 system (Valko *et al.* 2005: 2006). For instance, hydroxyl radical (OH•), regarded as the most reactive vis-à-vis other in-vivo ROS, is produced by Fenton reaction characterized by the reaction of $O_2^{\bullet-}$ with H_2O_2 and with Fe^{2+} or Cu^+ (Kumar and Pandey, 2015). As per nonenzymatic reactions, ROS is produced as a result of the following; reaction of oxygen with organic compound, cells being exposed to ionization radiations, as well as during mitochondrial respiration (Pizzano *et al.* 2017; Kumar and Pandey, 2015). Reactive oxygen species are generated both endogenously and exogenously. Although, when maintained at a low concentration, ROS has been shown to mitigate harmful bodies in the immune system, particularly in the synthesis of phagocytes for the destruction of pathogenic microbes (Willcox *et al.* 2004; Droge, 2002). However, at a significantly increased levels, ROS and free radicals leads to an oxidative stress; a phenomenon that has a deleterious effect on numerous cellular structures inter alia biological membrane, lipoproteins, and the nucleic acid (Pizzano *et al.* 2017; Young and Woodside, 2001). The emergence of oxidative stress is as a result of an imbalance between reactive oxygen species or free radical and the antioxidants, where the formation of these free radicals outweighs the free radical scavenging activities and ability of antioxidants (Pizzino *et al.* 2017). Additionally, oxidative stress has largely been identified as a key player that is implicated in both chronic, and degenerative diseases (Sato *et al.*, 2013; Pizzano *et al.*

2017) and it is considered as either a primary or a secondary etiology of several disease conditions (Pizzano *et al.* 2017). The study of Pacher *et al.* (2007) revealed that oxidative stress is implicated in cardiovascular diseases (CVDs) and it acts as a trigger of atherosclerosis (Pacher *et al.* 2007). Droge, (2002), reported that oxidative stress is a primary cause of a plethora of diseases affecting renal apparatus including renal failure, proteinuria, and uremia (Droge, 2002). Butterfield, (2002) reported that oxidative stress has been shown to be linked to neurological diseases such as Parkinson's disease, Alzheimer's disease (AD) and multiple sclerosis, (Halliwell, 2001) and also the study of Valko *et al.* (2004) demonstrated that oxidative stress damage and drives/or promote the mutation of DNA, chromosomal abnormalities and oncogene activation for cancer development (Valko *et al.* 2007). The body attempts to mitigate the deleterious effects of oxidative stress and free radicals by the use of antioxidants. A sizeable amount of scientific reports have shown that antioxidants play a crucial role in scavenging free radicals and relieving oxidative stress implicated in several disease conditions. In the light of this, chitosan, an amino polysaccharide produced from the deacetylation of chitin found in the shell of crustaceans, has extensively in recent times been shown to exhibit antioxidant activity both in vivo and in vitro (Avelelas *et al.* 2019; Anraku *et al.* 2011; Wölfle *et al.* 2014; Ali *et al.* 2019). Therefore, the present study examined the effect of chitosan on survival and oxidative stress 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 the 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: The processing of chitosan from the crustacean shells was based on the protocol described by (Randy *et al.* 2015). Chitosan was extracted from chitin-rich snail shells by the processes of deproteinization, deminerization and deacetylation. Deproteinization was done by boiling powdered shell with 4% weight/volume (w/v) NaOH for 2 hours. The samples were demineralized using 1% HCl with four times its quantity and 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. The extracted Chitosan was further subjected to treatment to achieve a dry whitish powdere, chitosan.

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 (7) treatment groups, with each group having five (5) vials, containing fifty (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 (Abolaji *et al.* 2014; Augustina *et al.* 2019).

Preparation of Tissue Homogenate: The flies were immobilized in ice and homogenized in 0.1 M phosphate buffer, pH 7.4. The resulting homogenates were centrifuged at 10,000 × g, at 4°C for 10 min in a Kenxin refrigerated centrifuge. Subsequently, the supernatant was separated from the pellet into labeled Eppendorf tubes and used for the various biochemical assays.

Biochemical Assay: Determination of Catalase (CAT): Catalase activity in the homogenate samples was determined according to the method of Shina (1972). In brief, 0.1 ml of each tissue homogenate sample was reacted with 0.4 ml 2 M H_2O_2 in the presence of 1.0 ml 0.01 M phosphate buffer (pH 7.0). The reaction was stopped by the addition of 2.0 ml dichromate acetic acid. The absorbance of the reaction mixture was taken at 620 nm in a spectrophotometer. A standard curve was prepared by reacting 0.4 mol of 2 M H_2O_2 with 2 ml dichromate acetic acid in the presence of 1.0 ml 0.01 M sodium phosphate buffer (pH 7.0). The catalase activity was thereafter calculated and expressed as unit/mg protein where 1 unit = 1 µmol of H_2O_2 consumed per minute.

Determination of Gluthathione-S-Transferase: This assay was carried out according to the method of Habig and Jakoby (1981). It involves the pre-incubation of reaction mixture containing 1.0 ml 100 mM phosphate buffer (pH 6.5), 30 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 0.7 ml of distilled water for 5 mins at 37°C. The reaction was started by the addition of 0.1 ml of the tissue homogenate and 0.1 ml 30 mM glutathione as substrate. The absorbance of the reaction mixture was monitored after 5 min at 340 nm in a Spectrophotometer. Reaction mixture without enzyme was used as a blank. The activity of GST was calculated and expressed as unit of GST activity per mg protein.

Determination of Gluthathione: This assay was carried out according to the method of Tietze and modified by Adams. It involves the oxidation of GSH by Ellman's reagent (5',5'-dithiobis-2-nitrobenzoic acid) resulting in the formation of GSSG and 5-thio-2-nitrobenzoic acid (TNB). GSSG is then reduced to GSH by gluthathione reductase using reducing equivalent provided by NADPH. The rate of TNB formation is proportional to the sum of GSH present in the sample and is determined by measuring the formation of TNB at 412nm using a spectrophotometer.

Statistical Analysis: Data were treated by ANOVA (analysis of variance) and mean separation was done using Turkey HSD and Duncan. Paired T-test was used to establish difference in timely events among same individual group animals and $\rho<0.05$ were considered significant. Data was expressed as means±standard deviation. All statistical analysis was done using IBM SPSS Version 22 and Microsoft Excel.

RESULTS AND DISCUSSION

Drosophila melanogaster flies were equally distributed in the study groups and were exposed to the same atmospheric condition and handling techniques for a period of 14 days. The control group was exposed to the normal feed, while the remaining groups were fed with varying concentrations of chitosan dietary inclusion 10µg, 20µg, 40µg, 320µg, 640µg, and 1mg respectively. Following the 14 days feeding, survival rate was determined by counting the number of active flies present in the respective groups, and the result of the present study as indicated in figure 1 revealed that, the group fed 320 µg chitosan had a higher survival rate with 23% relative the control group as well as the remaining treatment groups. It was also found that the other groups exposed to the following concentrations of dietary inclusion of chitosan; 10µg, 20µg, 40µg, 640µg, and 1mg, showed a higher survival rate with 20%, 22%, 15%, 10%, and 18% respectively in comparison to the control group as revealed in figure 1.

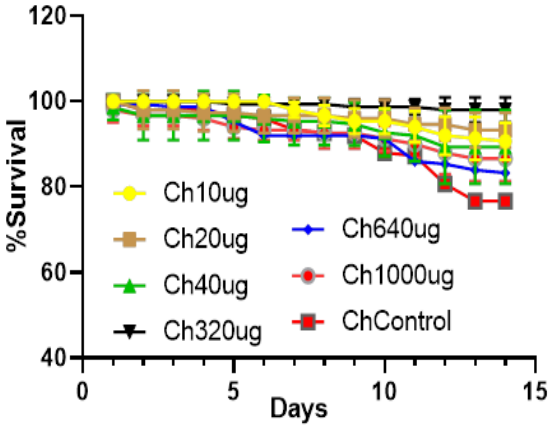

Fig 1: Survival curve for *Drosophila melanogaster* flies. Abbreviation denote Ch10ug: 10µg of Chitosan meal, Ch20ug: 20µg of chitosan meal, Ch40ug: 40µg of chitosan meal, Ch320ug: 320µg of chitosan meal, Ch640ug: 640µg of chitosan meal, Ch1mg: 1mg of chitosan meal, and Control: Control

Following a 14-day feeding of *Drosophila melanogaster* with dietary inclusion of chitosan at various concentration, oxidative stress markers were assessed. The result of the present study as shown in table 1 reveals that 10µg chitosan dietary inclusion resulted to a significantly $p<0.05$ decreased level of GSH (62.02±12.73) vis-à-vis the control (182.77±29.73). Conversely, there was no significant difference ($p>0.05$) in the levels of CAT and GST for 10µg chitosan fed group in comparison to the control group. Table 2 reveals that *Drosophila melanogaster* fed with 20µg chitosan dietary inclusion resulted to altered level of oxidative markers. It was observed in the table that the level of GSH for the group fed with 20µg chitosan dietary inclusion (70.35±7.22) was significantly $p<0.05$ lower relative to the control group (182.77±29.73) that was exposed to a standard feed for *Drosophila melanogaster*. However, there was no significant difference $p>0.05$ observed for CAT and

GST between the group fed with 20µg chitosan dietary inclusion and the control.

Table 1. Catalase, GST and GSH activities in in Control and flies fed with 10µg chitosan meal

Parameters	Control	10µg in chitosan meal	P-Value	Level
CAT (mg/dl)	0.852±0.13	0.82±0.15	0.39	Not significant
GST (mg/dl)	2.38±0.98	3.05±0.17	0.15	Not significant
GSH (mg/dl)	182.77±29.73	62.02±12.73	0.000344	Highly significant

Data are expressed as means ± SD. P<0.05 was considered Significant. Abbreviations denote CAT: Catalase, GST: Glutathione S-transferase. GSH: Glutathione

Table 2. Catalase, GST and GSH activities in in Control and flies fed with 20µg chitosan meal

Parameters	Control	20µg in chitosan meal	P-Value	Level
CAT (mg/dl)	0.852±0.13	0.70±0.28	0.99	Not significant
GST (mg/dl)	2.38±0.98	1.15±0.13	0.02	Not significant
GSH (mg/dl)	182.77±29.73	70.35±7.73	0.0016	Highly significant

Data are expressed as means ± SD. P<0.05 was considered Significant. Abbreviations denote CAT: Catalase, GST: Glutathione S-transferase. GSH: Glutathione

Table 3 shows that treatment with 40µg chitosan dietary inclusion resulted in the alteration of some of the oxidative stress markers that were examined. It was observed that the level of CAT (0.49±0.13) and GSH (70.59±13.01) in the group fed with 40µg chitosan dietary inclusion was significantly $p<0.05$ lower relative the control group CAT (0.852±0.13) and (182.77±29.73); whereas there was no statistical difference $p>0.05$ in the level of GST for the group fed 40µg chitosan dietary inclusion when compared with the control group. The effect of chitosan dietary inclusion at the concentration of 320µg on oxidative stress markers in *Drosophila melanogaster* was also determined. The result as shown in table 4 reveals that the CAT (0.62±0.079) and GSH (60.24±3.76) of the group fed with 320µg chitosan diet was significantly $p<0.05$ lower in comparison with the control group CAT (0.852±0.13) and GSH (182.77±29.73). Conversely, there was no significant difference $p>0.05$ in the level of GST.

Table 3. Catalase, GST and GSH activities in in Control and flies fed with 40µg chitosan meal

Parameters	Control	40µg in chitosan meal	P-Value	Level
CAT (mg/dl)	0.852±0.13	0.49±0.13	0.0025	Highly significant
GST (mg/dl)	2.38±0.98	2.98±2.21	0.32	Not significant
GSH (mg/dl)	182.77±29.73	70.59±13.01	0.0019	Highly significant

Data are expressed as mean ± SD. P<0.05 was considered Significant. Abbreviations denote CAT: Catalase, GST: Glutathione S-transferase. GSH: Glutathione.

Table 4. Catalase, GST and GSH activities in Control and flies fed with 320µg chitosan meal

Parameters	Control	320µg in chitosan meal	P-Value	Level
CAT (mg/dl)	0.852±0.13	0.62±0.079	0.021	Highly significant
GST (mg/dl)	2.38±0.98	1.67±0.31	0.11	Not significant
GSH (mg/dl)	182.77±29.73	60.24±3.76	0.0010	Highly significant

Data are expressed as mean±SD. P<0.05 was considered Significant. Abbreviations denote CAT: Catalase, GST: Glutathione S-transferase. GSH: Glutathione.

Chitosan is an amino polysaccharide, produced from the deacetylation of chitin obtained from the hard outer skeletons crustaceans and insects. Chitin, found in the shell of crustaceans, the cuticles of insects, and the cell walls of fungi, is the second abundant biopolymer in the nature (Knorr, 1984). Chitosan has so many uses in regards to medicine. Early research shows that rinsing with a chitosan mouth wash for 2 weeks helps to stop plaque from forming on the teeth (Sano *et al*., 2003). It has also help to make the eyes feel less dry in people who have normally dry eyes by using eye drops containing chitosan (Katrin *et al*., 2018). Early research has also shown that taking chitosan by mouth may reduce high cholesterol, help to correct anemia, and improve physical strength, appetite, and sleep with people with renal/kidney failure that are on hemodialysis (Shibeng *et al*., 2011). Research has also supported the use of chitosan to improve wound healing and help nerves to grow back by applying chitosan to skin graft and using gels that contains chitosan and other ingredients might increase healing after getting teeth pulled (Akshat *et al*., 2019). As observed in the present study, the survival study showed that dietary inclusion of chitosan to *Drosophila melanogaster* meals at the concentration of 10µg, 20µg, 40µg, 320µg. 640µg, 1mg resulted in fewer deaths in the treatment groups than control. This indicates that there was higher survival of flies fed with chitosan meals than the regular meal used to feed the flies in the control group, indicating a possible

longevity potential of chitosan. The survival of the flies may be linked to lower oxidative stress as a result of dietary chitosan inclusions. The result of the present study tends in agreement to a growing body of evidence stating that oxidative damage caused by reactive oxygen species are linked to aging process, impaired physiological functions, etiology of diseases and a decreased lifespan (Kregel and Zhang, 2007; Ioana *et al.* 2016; Liguori, *et al.* 2018). Additionally, a buildup of oxidative stress are implicated in critical aspects of aging processes as well as the loss of functional macromolecule (DNA, lipids and proteins) needed for a healthy physiological activity of growth and lifespan (Flatt, 2012; Kregel and Zhang, 2007; Al-Gubory *et al.* 2010).

As observed, *Drosophila melanogaster* fed with chitosan meals showed high survival chances; with the 320µg chitosan meal having the best effect on fly survival. The implication is that the 320µg could be the concentration the flies have a better adaptation to. Reduced antioxidant levels have been linked to accelerate aging and pathological conditions which may ultimately lead to death. Optimum levels of antioxidant have been reported to slow aging process and reduces risks of life threatening conditions (Poljsak *et al*., 2012). The higher activities of catalase and gluthathione in the groups fed with 10µg, 20µg, 40µg, 320µg, 640µg and 1mg of chitosan meal could be due to increased ROS level in the flies which signifies a condition of redox imbalance and oxidative stress. This might explain the elevation in these antioxidant enzymes as an adaptive response by the flies to the oxidative stress. Adaptive response here involve the ability on an organism to counteract cellular damages caused by free radicals, which has been identified in *D. melanogaster* (Abolaji *et al.,* 2017). Cellular macromolecules are protected primarily from free radicals by endogenous antioxidant molecules like Gluthathione (GSH), Catalase. GSH protects cells by neutralizing the reactive oxygen species by the reduction of the harmful peroxides while Catalase catalyze the reduction of H_2O_2 to H_2O and O_2 and thus protects the cells from the deleterious peroxidative effect of hydrogen peroxide. Therefore the increase in this antioxidant enzymes as a result of chitosan in meals could be associated with physiological adaptive mechanism of the flies due to increased ROS production.

Conclusion: Chitosan in meal may enhance survival rate in *Drosophila*. Nutritional modulation using 320µg concentration of chitosan in meal was found to be most suitable. Therefore, chitosan in Drosophila meal may be useful in ameliorating the effect of systemic oxidative stress induction.

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