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# Alpha-ketoglutarate attenuates toxic effects of sodium nitroprusside and hydrogen peroxide in *Drosophila melanogaster*

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ABSTRACT

The protective effects of dietary alpha-ketoglutarate (AKG) are described that aid fruit flies, *Drosophila melanogaster*, to resist sodium nitroprusside (SNP) and hydrogen peroxide toxicity. Food supplementation with 10 mM AKG alleviated toxic effects of 1 mM SNP added to food and improved fly development. Dietary AKG also prevented the increase in levels of oxidative stress markers seen in SNP-reared adult flies. *In vitro* AKG did not affect the rate of SNP decomposition and did not bind iron and nitrite ions released in this process. Alpha-ketoglutarate also displayed high $H_2O_2$-scavenging activity *in vitro* and efficiently protected adult flies against this compound in combined treatments. Based on the observed antioxidant activity of AKG, it may be suggested that the antioxidant mode of AKG action (apart from its cyanide-binding capability) may be used to prevent the toxic effects of SNP and improve general physiological state of *D. melanogaster* and other animals and humans.



## 1. Introduction

Alpha-ketoglutarate (AKG) is an important intermediate in the Krebs cycle that bridges cellular energy metabolism with the metabolism of amino acids as well as acting as an important nitrogen transporter in metabolic pathways (Harrison and Pierzynowski, 2008). Studies using animal models have shown that dietary supplementation with AKG has favorable effects on energy status, protein synthesis, growth, and performance (Junghans et al., 2006; Hou et al., 2011; Dobrowolski et al., 2013; Yao et al., 2012) and can potentially prevent the development of several systemic diseases commonly found in the elderly (Kjellman et al., 1997; Harrison and Pierzynowski, 2008; Niemiec et al., 2011; Radzki et al., 2012). Protective properties of AKG were also demonstrated in hyperammonemia, hyperaminoaciduria, or exposures to toxic nitrogen chemicals such as cyanide, ammonia, ammonium compounds, amines, and hydrazines (Bhattacharya et al., 2002; Dakshayani et al., 2006; Bhattacharya et al., 2009). Furthermore, AKG was found to be an effective oral treatment at poisoning with cyanide or cyanogen-containing compounds (Bhattacharya et al., 2009, Mitchell et al., 2013). Cyanide is a mitochondrial toxin that impairs cellular respiration due to inhibition of cytochrome oxidase, a terminal enzyme of mitochondrial electron transport chain (Pettersen and Cohen, 1993). Treatment with cyanide was found to inhibit multiple enzymes including antioxidant ones (particularly superoxide dismutase), and oxidative stress is one such event which has been frequently associated with cyanide toxicity (Ardelt et al., 1994; Tulsawani et al., 2005; Tulsawani and Bhattacharya, 2006; Mathangi et al., 2011). The protective effects of AKG are considered to be due to the capability of the carbonyl groups on AKG to interact with cyanide to yield less toxic cyanohydrin intermediates (Niknahad et al., 1994, Mitchell et al., 2013). In the line with this, the potential of AKG to prevent depletion of antioxidant defenses or counteract the increase in levels of oxidative stress markers during acute or sub-chronic cyanide poisoning was demonstrated (Tulsawani et al., 2005; Tulsawani and Bhattacharya, 2006; Bhattacharya et al., 2009; Mathangi et al., 2011). Furthermore, several studies reported that the protective effects of AKG against various toxic agents *in vitro* and *in vivo* could be related with its direct antioxidant action (Sokołowska et al., 1999; Murugesan and Subramanian, 2006; Kovalenko et al., 2011; Niemiec et al., 2011; Long and Halliwell, 2011). In view of this, an antioxidant

*Abbreviations:* AKG, alpha-ketoglutarate; ETC, electron transport chain; H-SH, high molecular mass thiol groups; L-SH, low molecular mass thiol groups; NOS, NO-synthase; ROS, reactive oxygen species; TCA, trichloroacetic acid; LOOH, lipid peroxides; SNP, sodium nitroprusside.

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action of AKG could also be involved in the attenuation of toxicity by cyanide and cyanogens.

Sodium nitroprusside (SNP), $Na_2[Fe(CN)_5NO]$, is one of the best known cyanogens and is widely used clinically as a vasodilator for the treatment of cardiac failure and hypertension due to its short half-life in solution and its ability to release nitric oxide (•NO) (Hottinger et al., 2014). However, SNP decomposition also leads to the release of cyanide and iron moieties (Thomas et al., 2009; Bhattacharya et al., 2009; Lozinsky et al., 2012). Treatment with SNP is known to result in development of nitrosative and oxidative stresses in different model organisms (Lushchak and Lushchak, 2008; Semchuk et al., 2011; Lozinsky et al., 2012; Park et al., 2012), and several mechanisms have been proposed to be involved in SNP-induced enhancement of the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lushchak and Lushchak, 2008; Lozinsky et al., 2012).

In recent years, the fruit fly *Drosophila melanogaster*, a popular genetic model, has begun to be used extensively in toxicological and nutritional research. Studies with *Drosophila* have demonstrated conservation of regulatory mechanisms involved in metabolic homeostasis under different nutritional interventions or drug treatments (Kaun et al., 2012; Lozinsky et al., 2012, 2013; Abolaji et al., 2014; Rovenko et al., 2014, 2015; Perkhulyn et al., 2015). We recently showed that food supplementation with SNP had toxic effects on *D. melanogaster* causing a developmental delay and reducing the number of adult flies eclosed (Losinsky et al., 2012). The adverse effects of SNP were partly diminished by use of the protonophore, 2,4-dinitrophenol, which restored redox homeostasis in flies; that implicated oxidative/nitsosative stress involvement in SNP-induced toxicity (Losinsky et al., 2013). Taking into account the fact that AKG can act as antidote for cyanide poisoning and exhibits antioxidant activity, the present study aimed to examine the possible protective effects of dietary AKG against SNP-induced toxicity in *D. melanogaster* at larval and adult stages. To determine whether beneficial effects of AKG could be connected with its antioxidant properties, the effects of AKG on the resistance of adult flies to oxidative stress induced by hydrogen peroxide were tested as well as the ability of AKG to scavenge $H_2O_2$ *in vitro*.

## 2. Methods and materials

### 2.1. Flies and rearing

Wild-type Canton S and mutant $w^{1118}$ flies were used in experiments. Stock flies were kindly provided by Bloomington Stock Center (Indiana University, USA). All fly cultures were kept at 25 °C, 55–60% humidity in a 12-h dark/light cycle. Parental populations of flies were maintained on yeast–corn–molasses media containing 7.5% (v/v) molasses, 5% (w/v) yeast, 6% cornmeal (w/v), 1% (w/v) agar, and 0.18% (w/v) methylparaben to inhibit mold growth (Rovenko et al., 2014). For experiments, flies were reared starting from the egg stage on a medium containing 5% sucrose, 5% yeast, 1% agar, and 0.18% methylparaben and this group was used as the control. Experimental cohorts of flies were cultivated on the same food but supplemented with 1 mM SNP, 10 mM alpha-ketoglutaric acid disodium salt (AKG) or a mixture of 1 mM SNP and 10 mM AKG (pH 5.3–5.5). About 100 eggs laid by parental flies within a 6-h time period were put in each 100 ml glass bottle with 15 ml of the experimental diets.

The speed of development of flies on the different experimental diets was assessed by counting of the number of pupae formed once per day, starting 96 h after egg deposition. The total number of pupae formed at day 10 was set as 100% and the pupation at each day was expressed as the percentage of pupae formed over this time. Developmental survival was also assessed as the percentage of eggs that were able to reach the pupa stage.

Newly eclosed flies were transferred into flasks with the respective experimental foods and held for 2 days. Furthermore, 2-day-old flies were mildly anesthetized with carbon dioxide gas, separated by sexes, and then used for physiological tests or quickly frozen in liquid nitrogen for further biochemical analysis.

### 2.2. Effect of test chemicals on larval behavior (pupation height preference)

Changes in larval behavior in response to the experimental chemical exposures were assessed as pupation height preference. Before pupation, larvae climb up the sides of the vials, attach themselves and then pupate. When all larvae had pupated, the distance from the food surface to the place of attachment of each pupa was measured (pupation on the food surface was scored as zero). The pupation height was expressed in millimeters (mm) as described previously (Lozinsky et al., 2012).

### 2.3. Assay of food intake

Food intake was measured as described previously by Lushchak et al. (2011). Briefly, groups of 15 third instar larvae reared on the control media were placed for 20 min on control and experimental diets (in Petri dishes), which also contained 0.5% FD&C Blue No. 1 dye. After feeding, larvae were homogenized in 50 mM potassium phosphate buffer (KPi, pH 7.0) at a ratio 1:100 (mg/μl) and centrifuged at room temperature at 13,000 g for 15 min. Supernatant samples were removed and absorbance was measured at 629 nm and compared against a calibration curve built with different concentrations of the dye.

### 2.4. Testing of resistance of adult flies to SNP and $H_2O_2$

Ten 2-day-old flies reared on the control food or the diet with 10 mM AKG were transferred into empty vials for 2 h of starvation. After starvation, flies were transferred into vials containing folded and rammed strips (2.4 × 12 cm) of four-layer cellulose filter paper soaked with 0.8 ml of 5% sucrose solutions additionally containing (1) 1 mM SNP, (2) a mixture of 1 mM SNP and 10 mM AKG, (3) 1 M $H_2O_2$, (4) a mixture of 1 M $H_2O_2$ and 10 mM AKG, or (5) a mixture of 1 M $H_2O_2$ and 1 M AKG. Survivors were counted at defined time intervals over 72 h of exposure and values were expressed as the percentage of flies that survived.

### 2.5. Assay of $H_2O_2$ scavenging activity of AKG

To assess the $H_2O_2$ scavenging activity of AKG, mixtures containing 10 mM $H_2O_2$, and different concentrations of AKG were prepared in 50 mM KPi (pH 7.0) and incubated for 30 min at 28 °C; controls omitted AKG. After incubation, the samples were diluted 200 times and 200 μl aliquots were mixed with 1.8 ml FOX reagent (0.25 mM $FeSO_4$, 25 mM $H_2SO_4$, 0.1 mM xylenol orange and 100 mM sorbitol) as previously described (Nourooz-Zadeh et al., 1994). The reaction mixture was then incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 580 nm. The percentage of hydrogen peroxide scavenged by AKG was calculated as follows: % Scavenged $[H_2O_2] = [(A_0 - A_1)/A_0] \times 100$, where $A_0$ was the absorbance of the control and $A_1$ was the absorbance in the presence of AKG.

### 2.6. Assay of iron and nitrite levels in solutions with SNP and AKG

Water solutions of SNP are unstable and SNP decomposes rapidly releasing nitric oxide (•NO), iron ($Fe^{2+}$) and cyanide ($CN^-$)

moieties (Bhattacharya et al., 2009; Hottinger et al., 2014). Determination of the products of 1 mM SNP degradation was performed in 5% sucrose solution (used to determine SNP toxicity in adults flies) without or with addition of 10 mM AKG. The content of iron ions was determined by reaction with 1,10-phenanthroline (Lee et al., 1948). The method is based on the formation of orange–red complex by the interaction of iron ions (II) with 1,10-phenanthroline with absorbance of the complex measured at 510 nm. A volume of 0.5 ml of 1 mM SNP in 5% sucrose was mixed with 0.5 ml of 10% hydroxylamine hydrochloride and 0.5 ml of 0.5% 1,10-phenanthroline, and then incubated for 1 h at room temperature. To build the calibration curve a solution of $FeNH_4(SO_4)_2 \bullet 12H_2O$, containing 5 μg of iron in 1 ml was used.

Nitric oxide was quantified as its stable oxidized form, nitrite, determined by the Griess reaction (Privat et al., 1997). Aliquots of 1 mM SNP solution in 5% sucrose were mixed with 1% Griess reagent and incubated for 15 min. The absorbance at 540 nm was measured, and the nitrite concentration was determined using a calibration curve made with sodium nitrite standards in a range from 0.25 to 2.0 μg. The values were expressed as micrograms $NO_2^-$ per ml of solution.

### 2.7. Assay of chelating properties of AKG

The 1,10-phenanthroline method described above (Lee et al., 1948) was used to examine the iron chelating properties of AKG. The mixture containing freshly prepared $FeSO_4$ (150 μM) and different concentrations of AKG was allowed to react for 5 min at room temperature. After that, 1,10-phenanthroline solution was added to determine free $Fe^{2+}$, which did not react with AKG. The absorbance was measured at 510 nm, and EDTA was used as positive control. The values were expressed as a percentage of the control determined in the absence of AKG.

### 2.8. Preparation of homogenates of adult flies for metabolic analyses

Frozen flies were weighed and homogenized using a Potter-Elvehjem glass homogenizer in lysis buffer (50 mM KPi, pH 7.0, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA) in a 1:10 ratio (mg flies:μl buffer). After centrifugation at 16,000 g for 15 min at 4 °C in an Eppendorf 5415 R centrifuge (Germany), the supernatants were collected and used for different assays using Spekol 211 (Carl Zeiss Jena, Germany) or SF-46 (LOMO, USSR) spectrophotometers.

### 2.9. Assays of catalase activity, nitrite and total iron levels, and protein concentration

Catalase activity was measured by monitoring the dismutation of hydrogen peroxide at 240 nm using the extinction coefficient for hydrogen peroxide of 39.4 $M^{-1}$ $cm^{-1}$ (Aebi, 1984). The activity of catalase was assayed in 2 ml of medium containing 50 mM KPi (pH 7.0), 0.5 mM EDTA, 10 mM $H_2O_2$, and 3 μl of supernatant.

Total iron and nitrite levels in fly bodies were determined as described above by the 1,10-phenanthroline method (Lee et al., 1948) and the Griess reaction (Privat et al., 1997), respectively. Values were expressed as micrograms per milligram of wet mass.

Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method (Bradford, 1976) with bovine serum albumin as a standard.

### 2.10. Assay of oxidative stress markers

Free thiols were measured spectrophotometrically by the Ellman procedure with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm (Ellman, 1959). Fly homogenates were prepared, centrifuged and supernatants saved, and then total low molecular mass and high molecular mass thiol contents were measured as was described previously (Lushchak and Bagnyukova, 2006). Briefly, the sum of soluble low and high molecular mass thiol-containing compounds was measured in the supernatants. Then, to determine the non-protein low molecular mass thiols (L-SH), trichloroacetic acid TCA was added to supernatants to a final concentration of 10% followed by centrifuging; supernatants from this step were used to assay low molecular mass thiols. High molecular mass thiol (H-SH) content was calculated by subtraction. Thiol concentrations were expressed as micromoles of SH-groups per gram wet mass.

Lipid peroxide (LOOH) content was assayed with xylenol orange (Hermes-Lima et al., 1995) with minor modifications (Lushchak et al., 2005). For this, flies were homogenized 1:20 w:v in 96% cold (~5 °C) ethanol, centrifuged for 5 min at 13,000 g, and supernatants were used for assay. The content of LOOH was expressed as micromoles of cumene hydroperoxide equivalents per gram of wet mass.

### 2.11. Statistical analysis

Experimental data are expressed as the mean value of four—six independent experiments ± the standard error of the mean (SEM). Statistical analysis was performed using a two-tailed Student's *t*-test or analysis of variance (ANOVA) followed by the two-tailed Dunnett's test or Student–Newman–Keuls (SNK) test. Experimental data were calculated using the Microsoft Excel computer program (Microsoft Corporation, Redmond, WA, USA).

## 3. Results and discussion

### 3.1. Dietary AKG reduces developmental toxicity of SNP on D. melanogaster without influence on food intake

Recently we found that SNP had deleterious effects on *D. melanogaster* development and that tolerance to this toxicant depended on the concentrations used and fly strain specificity (Lozinsky et al., 2012, 2013). Given these findings, we became interested in the protective ability of dietary AKG against SNP-induced larval toxicity and the present study evaluates this in two strains of *D. melanogaster*, Canton S, and its derivative strain $w^{1118}$. The concentration of SNP used in this study was one that effectively reduced the pupation rate of the two strains, as reported earlier (Lozinsky et al., 2012). Fig. 1 shows the effect on development, assessed by pupation speed, when fruit fly larvae were raised on media supplemented with 1 mM SNP alone, 10 mM AKG alone, or SNP + AKG, as compared with controls. In both Canton S and $w^{1118}$, strains consumption of food containing SNP-delayed pupation (Fig. 1A, B) and reduced the total number of pupae formed (Fig. 1C). A delay in the transition from larva to pupae for larvae fed SNP was seen in both strains but was more pronounced in the $w^{1118}$ strain (Fig. 1B) with the median time to pupation for larvae raised on diets with 1 mM SNP being 13 and 32% higher in Canton S and $w^{1118}$ strains, respectively, compared to controls (Fig. 1D). The total percentage of larvae that pupated did not significantly differ between the control cultures of the two strains (96 ± 3% in Canton S *versus* 87 ± 4% in $w^{1118}$) and decreased in both strains on food supplemented with 1 mM SNP (Fig. 1C). The reduction in total yield of pupae from SNP-fed larvae was similar in both strains and was a decrease to about 50% of control values confirming developmental toxicity of SNP in flies, as previously reported (Lozinsky et al., 2012). Flies reared on food supplemented with a mixture of 1 mM SNP and 10 mM AKG displayed only about a 25% reduction in developmental mortality compared with controls, a significantly reduced mortality

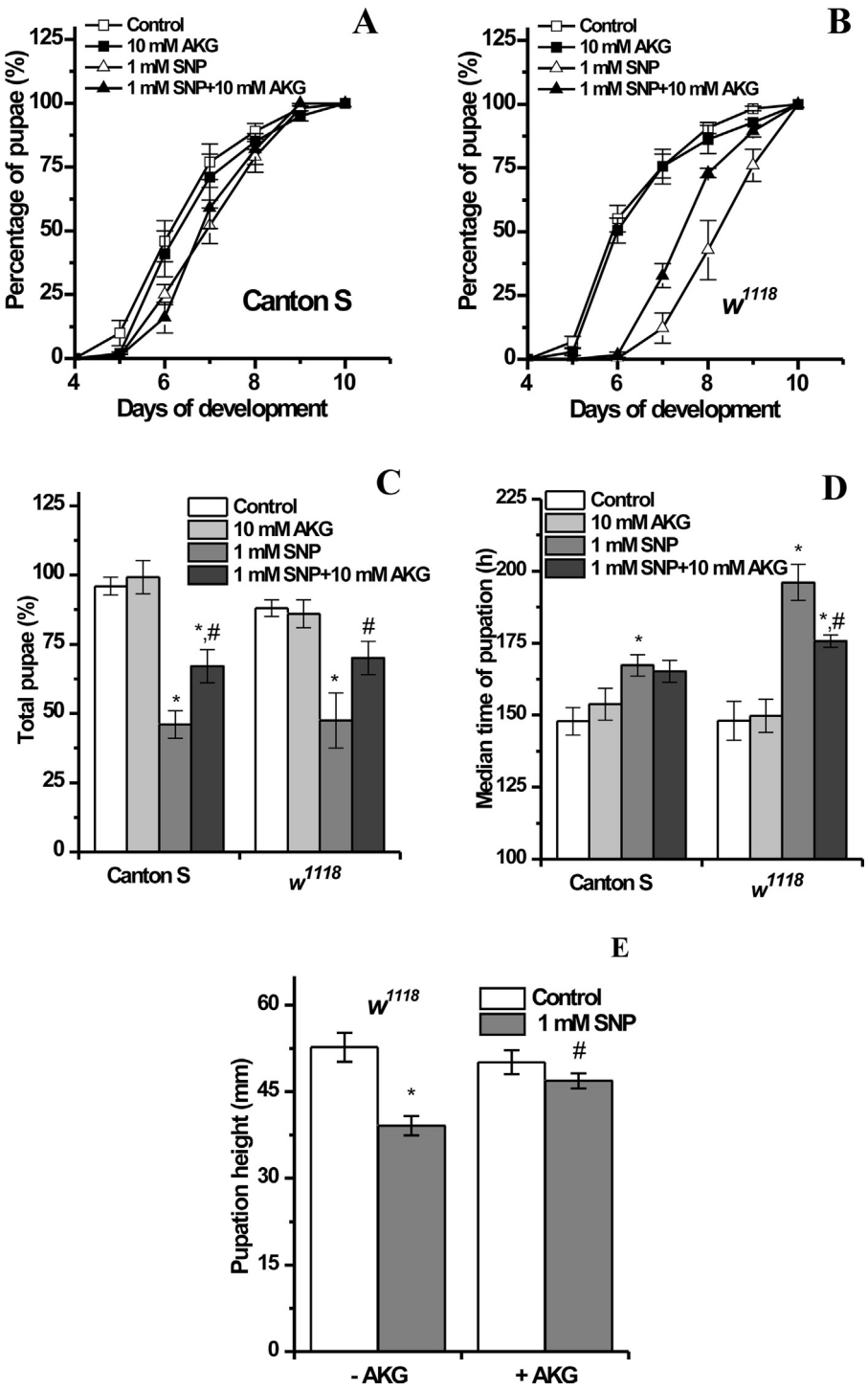


**Fig. 1.** Development pattern of *D. melanogaster* flies reared on yeast-sucrose medium or on diets supplemented with 10 mM AKG, 1 mM SNP, or both SNP + AKG. Graphs show percentage of larvae that pupated over time in Canton S (A) and $w^{1118}$ (B) strains, percentage of eggs pupated (C), median time of fly development to pupation (D), and pupation height in the $w^{1118}$ strain (E). Data are means ± SEM, $n = 4$–6. *Significantly different from the corresponding control group or #from the SNP-fed group, $P < 0.05$ as determined by ANOVA and the *post hoc* Student–Newman–Keuls test.

compared with larvae reared on food containing SNP alone. Accordingly, combined food supplementation with SNP and AKG allowed 70% eggs to make the transition through the larval stage into pupae *versus* just 45% of those which developed on SNP only. However, AKG did not alter the pupation delay seen for SNP-exposed Canton S larvae, but decreased the median time to pupation by 11% for the $w^{1118}$ strain on the medium with SNP (Fig. 1 D). In addition, AKG supplementation had a positive impact on pupation height, one of the widely used parameters to determine toxicity of various chemicals in flies reared on solid food (Lozinsky et al., 2012). Flies reared on SNP diet showed a decrease in pupation height by 35% as compared with control values but the presence of AKG restored this parameter to initial values of control flies (Fig. 1 D). Measurements of food intake showed that AKG did not affect consumption of food by larvae (Table 1), but larvae consumed about one-third less food when raised on a diet containing 1 mM SNP or a mixture of 1 mM

**Table 1**
Food consumption by larvae and body mass of 2-day-old *D. melanogaster* and reared on control food or on food supplemented with SNP, AKG, or mixture of SNP and AKG.

| Conditions | Food consumption by Canton S larvae, μg/20 min/larva | Body mass, μg | | | |
|---|---|---|---|---|---|
| | | Canton S | | $w^{1118}$ | |
| | | Males | Females | Males | Females |
| Control | 36 ± 7 | 795 ± 26 | 1361 ± 66 | 753 ± 24 | 1244 ± 33 |
| 10 mM AKG | 40 ± 7 | 793 ± 13 | 1352 ± 51 | 726 ± 15 | 1235 ± 61 |
| 1 mM SNP | 24 ± 2* | 778 ± 16 | 1090 ± 29* | 809 ± 10 | 1186 ± 31 |
| 1 mM SNP + 10 mM AKG | 22 ± 2* | 764 ± 80 | 1070 ± 40* | 771 ± 49 | 1117 ± 46 |

Data are presented as means ± SEM, $n = 5$–8. *Significantly different from control group with $P < 0.05$ using ANOVA and the *post hoc* Dunnett's test for multiple comparisons.

SNP and 10 mM AKG. A lower feeding rate on diets containing SNP might be considered as an adaptive mechanism of larvae to these stressful conditions. It is established that if there are toxic compounds in food that require energy for detoxification that larvae can maximize their energy intake by slowing their feeding rates and at the same time increase efficiency of food use (Mueller and Barter, 2015). The body masses of 2-day-old Canton S and $w^{1118}$ males as well as $w^{1118}$ females developed on SNP diets did not differ from those of corresponding controls, but SNP-fed Canton S females had 25% lower body masses compared with control females, as did those raise on SNP + AKG (Table 1). Food supplementation with AKG alone did not affect fly body mass in any group. The lower body masses of flies reared on food supplemented with SNP or SNP with AKG might result from insufficient accumulation of fuel reserves during the larval stage likely due to decreased food consumption.

In general, the results demonstrate that exogenous AKG can partly alleviate toxic effects of SNP on development of *D. melanogaster* increasing both pupation rate and the total number of pupae formed. Since the addition of AKG to diet containing SNP did not enhance food consumption by larvae, this may suggest that AKG does not directly detoxify SNP in the medium.

### 3.2. Dietary AKG enhances survival of adult flies upon SNP exposure

The experiments above established that AKG reduced larval mortality for two strains of *D. melanogaster* raised on medium supplemented with SNP. Next we addressed the question of whether exogenous AKG was able to protect adult flies against SNP toxicity. Generally, fly resistance to different chemicals is assessed by monitoring the survival of flies for 1–3 days upon treatment with lethal concentrations of stressors (Schriner et al., 2012). In our case, the use of 1 mM SNP, which showed lethal effects at the larval stage, had no lethality for adult flies for 5 days (not shown). Therefore, we tested the effects of other SNP concentrations (5 and 10 mM) on viability of adult flies; as a result a concentration of 10 mM was chosen for further study since that caused significant mortality of flies after 3 days of incubation. To check the antidote activity of AKG against SNP-, we took 2-day-old $w^{1118}$ flies (raised on control diet or AKG supplemented diet) and transferred them to vials containing filter paper saturated with 10 mM SNP or a mixture of 10 mM SNP and 10 mM AKG in 5% sucrose solution. To exclude effects of sucrose on fly viability, the control group consisted of flies kept on 5% sucrose throughout the 5 day course; all flies in this group remained alive over the 5 days of treatment (data not shown). Hence, four fly groups were tested (Fig. 2): flies reared on control food and then transferred to vials containing 10 mM SNP ("Control"), or a mixture of 10 mM SNP and 10 mM AKG ("AKG-incubation") in 5% sucrose solution; flies reared on AKG-containing diet and then transferred to vials containing 10 mM SNP (AKG-rearing), or a mixture of 10 mM SNP and 10 mM AKG ("AKG-rearing and incubation") in 5% sucrose. Survival of control flies significantly decreased with time when held on medium containing 10 mM SNP, and males (Fig. 2A) were more sensitive to SNP than females (Fig. 2B). The percentage of flies alive after 24, 48, and 72 h of exposure was 71, 61, and 53% in the case of control males and 93, 83, and 77% for control females, respectively. Surprisingly, flies that were reared as larvae on AKG showed higher susceptibility to SNP compared to the control counterparts, and only 37% of males and 59% of AKG-reared females survived the 72 h of exposure to 10 mM SNP. Combined exposure of control flies to both SNP and AKG did not significantly influence their survival, but increased viability of AKG-reared flies to control levels. It seems that AKG has less of a protective effect on adult flies exposed to SNP than was seen in the larval stage. At the same time, the concentration of SNP used for exposure of adult flies was 10-fold higher than for larvae, while the concentration of AKG was the same (10 mM). Thus, in the case of adult flies, the concentration of AKG could simply be not enough to provide a protective effect against SNP. Regardless of culturing conditions, females were more resistant to SNP than males. These results were not surprising, because female flies

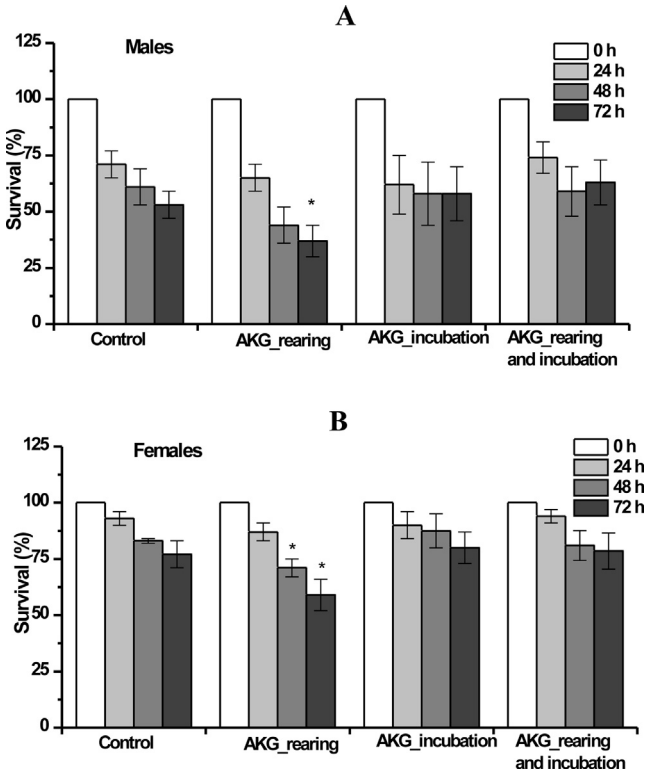


**Fig. 2.** Survival of 2-day-old *D. melanogaster* $w^{1118}$ male (A) and female (B) flies over the subsequent 3 days after transfer to adult diets containing 10 mM SNP. Conditions were: control—eggs raised on control diet and exposed to SNP in adult food; AKG-rearing—eggs reared on diet containing 10 mM AKG and exposed as adults to SNP in food; AKG-incubation—eggs raised on control diet and exposed to AKG and SNP in adult food; AKG-rearing and incubation—eggs reared on diet containing 10 mM AKG and exposed as adults to SNP + AKG in adult food. Data are means ± SEM, $n = 5$. *Significantly different from control group with $P < 0.05$ using Student's *t*-test.

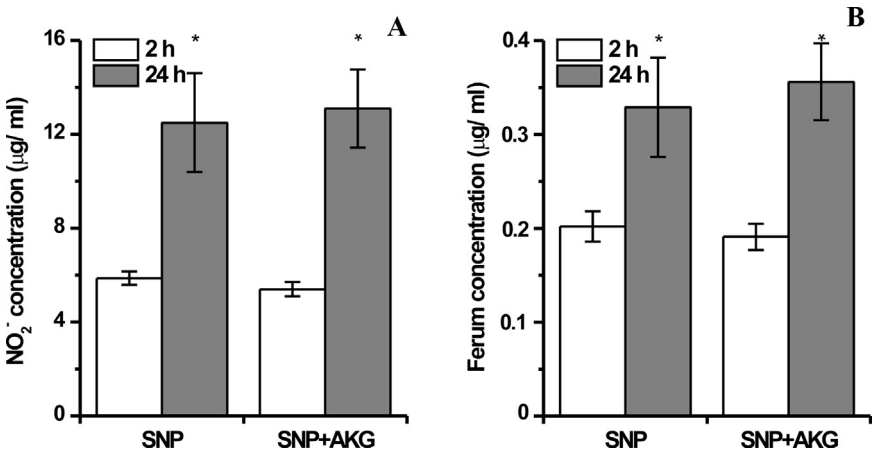


**Fig. 3.** Concentrations of nitrite ($NO_2^-$) (A) and total iron ions (B) in 1 mM SNP solution in 5% sucrose after incubation at 25 °C for 2 or 24 h in the presence or absence of 10 mM AKG. Data are means ± SEM, $n = 4$. *Significantly different from values at 2 h of incubation with $P < 0.05$ using Student's $t$-test.

evolutionarily developed higher resistance to various stresses, since they are responsible for generation of healthy offspring (Gospodaryov et al., 2013).

Earlier it was found that SNP had more toxic effects on cells and tissues with higher metabolic activity or with active functioning mitochondria (Pettersen and Cohen, 1993; Tulsawani and Bhattacharya, 2006; Bhattacharya et al., 2009). On other hand, dietary AKG was shown to be quickly metabolized after administration leading to intensification of metabolic processes in the body of experimental animals (Junghans et al., 2006; Hou et al., 2011; Dobrowolski et al., 2013; Yao et al., 2012). In particular, exogenous AKG can be incorporated into the Krebs cycle where it is oxidatively decarboxylated to succinyl-CoA yielding NADH, which, in turn, is used in mitochondrial electron transport chain (ETC) to produce ATP. Therefore, it can be supposed that flies developed on the medium with AKG might have higher intensity of metabolism, in particular, energetic processes (Hou et al., 2011). Stimulation of energy-producing processes clearly should be related to active function of mitochondria. The latter can increase the steady-state level of ROS as by-products of mitochondrial respiratory metabolism (Lushchak, 2014). In turn, an increment in ROS levels can trigger signaling pathways involved in induction of antioxidant defense. The mechanisms of SNP toxicity include an inhibition of antioxidant enzymes and cytochrome oxidase, which is the terminal enzyme of the mitochondrial ETC (Pettersen and Cohen, 1993; Bhattacharya et al., 2009). Therefore it can be supposed that SNP can block the ETC in active mitochondria in parallel with a marked inhibition of antioxidant defense in AKG-reared flies. As a result, a significant increase in ROS production could occur placing AKG-reared flies under more severe oxidative stress than their control counterparts (Lushchak, 2014). In this case, AKG incorporation in fly metabolism may enhance SNP toxicity. This assumption may explain, at least partly, the higher sensitivity of AKG-reared flies to SNP in our experiment. It also seems that exogenous AKG displays protective effects only when simultaneously administered with SNP, since pretreatment with AKG did not prevent toxic effects of SNP due to fast AKG metabolism.

### 3.3. Alpha-ketoglutarate does not influence level of nitrite and iron ions in SNP solution

Sodium nitroprusside is a very unstable compound in water solutions and it quickly decomposes to release end products such as nitric oxide, iron, and cyanide moieties; all of these compounds are supposed to be responsible for SNP toxic effects (Bhattacharya et al., 2009; Lozinsky et al., 2012; Hottinger et al., 2014). In *Drosophila* studies, supplementation of medium with SNP increased total iron content and the level of nitrite ions (as the stable oxidized form of •NO) in the culture medium as well as in the body of *D. melanogaster* larvae (Lozinsky et al., 2012). Under physiological conditions, nitric oxide is produced by NO-synthase (NOS) and plays an important role in coordination of metabolism, growth, and fly development (Cáceres et al., 2011), whereas at higher concentrations, •NO might directly inactivate NOS (Canteros et al., 1996). Therefore, it was suggested that •NO released at high-SNP concentrations inactivated NOS and altered developmental timing (Lozinsky et al., 2012). In addition, induction of oxidative/nitrosative stress due to SNP decomposition could be responsible for disruption of larval development (Lozinsky et al., 2012, 2013). In particular, after •NO release, the iron moiety of SNP may provide free iron coordination sites for $H_2O_2$ and their interaction could trigger the generation of highly

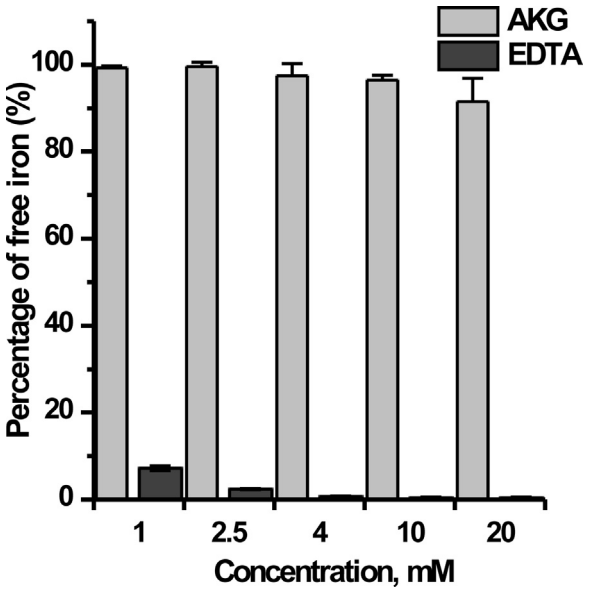


**Fig. 4.** Iron-chelating properties of AKG: 150 μM $FeSO_4$ and different concentrations of AKG or EDTA were allowed to interact for 15 min at room temperature. Then 1,10-phenanthroline solution was used to determine free $Fe^{2+}$. Spectrophotometric absorbance of the complex between free $Fe^{2+}$ and 1,10-phenantroline in the absence of AKG was set to 100%. Data are means ± SEM, $n = 5$.

**Table 2**
Selected characteristics of ROS homeostasis in 2-day-old Canton S females reared on control food or on diets supplemented with SNP or a mixture of SNP and AKG.

| Conditions | Catalase, U/mg protein | L-SH, μmol/g wet mass | H-SH, μmol/g wet mass | LOOH, μmol/g wet mass |
|---|---|---|---|---|
| Control | 225 ± 21 | 0.84 ± 0.10 | 4.12 ± 0.51 | 4.73 ± 0.66 |
| 1 mM SNP | 97 ± 12* | 0.86 ± 0.06 | 4.0 ± 0.32 | 7.19 ± 0.77* |
| 1 mM SNP + 10 mM AKG | 142 ± 14* | 0.75 ± 0.08 | 4.12 ± 0.21 | 3.14 ± 0.93 |

Data are presented as the means ± SEM, $n = 6–7$. *Significantly different from control group with $P < 0.01$ using ANOVA and the *post hoc* Dunnett's test for multiple comparisons.

reactive ROS, such as hydroxyl radicals (HO•) *via* the Fenton reaction (Hospodar'ov and Lushchak, 2004; Bagnyukova et al., 2006). In agreement with this, it was recently found that the protonophore 2.4-dinitrophenol could partly abolish the toxic effects of SNP *via* restoration of redox homestasis in flies (Lozinsky et al., 2013). Taking these facts into account, we further investigated whether the protective effect of AKG against SNP treatment in *D. melanogaster* could be connected with modulation of SNP decomposition. For this, we measured levels of total iron and nitrite ions by incubation of 1 mM SNP in 5% sucrose in the presence or absence of AKG. When nitric oxide (•NO) is exposed to an oxygen-rich aqueous environment, it is quickly oxidized to nitrite and/or nitrate anions. An assay using the Griess reaction was designed to detect nitrite, and thereby give an indirect assay for •NO production (Privat et al., 1997). In this system, total iron and nitrite levels in the medium rose in a time-dependent manner (Fig. 3) suggesting SNP decomposition. Interestingly, addition of AKG to the SNP solution did not affect the levels of total iron and nitrite ions. AKG was previously reported to manifest iron chelating properties (Puntel et al., 2005). However, we did not detect chelating ability of AKG toward free iron (II) by testing a broad range of AKG concentrations, in contrast to the effects of EDTA, a well-known iron-chelating compound (Fig. 4). Thus, it appears that AKG cannot slow SNP decomposition and is unable to bind free iron. On the other hand, AKG can interact directly with cyanide ions, another toxic product of SNP decomposition, to yield less toxic cyanohydrin intermediates that are easily metabolized (Niknahad et al., 1994, Mitchell et al., 2013). Obviously, the cyanohydrin-forming pathway might be involved in the AKG-protective action on *D. melanogaster* larvae against SNP-induced toxicity. In addition, SNP is more stable in solid food, but it may be decomposed quickly in the digestive tract of flies. Therefore, AKG may act as antidote for SNP toxicity in the fly bodies.

### 3.4. Alpha-ketoglutarate prevents oxidative damages in SNP-reared flies

As it was mentioned above, oxidative stress development can be responsible, at least partly, for toxic SNP effects. Several mechanisms may be implicated in SNP induction of oxidative stress, including inhibition of cytochrome oxidase by cyanide leading to increased ROS production by the mitochondrial electron transport chain (Pettersen and Cohen, 1993; Ardelt et al., 1994; Tulsawani and Bhattacharya, 2006; Mathangi et al., 2011). Iron cation and •NO released from SNP are also well-known inducers of oxidative stress in many organisms (Hospodar'ov and Lushchak, 2004; Bagnyukova et al., 2006; Lushchak and Lushchak, 2008; Semchuk et al., 2011; Lozinsky et al., 2012). In several studies, the ability of AKG to diminish the intensity of oxidative stress induced by cyanide was demonstrated and this was attributed to the cyanine-binding activity of AKG (Tulsawani et al., 2005; Bhattacharya et al., 2009; Mathangi et al., 2011). At the same time, antioxidant properties of AKG both *in vivo* and *in vitro* were reported, and in particular, AKG was found to be involved in the prevention of lipid peroxidation in rats under chronic ethanol administration (Velvizhi et al., 2002), sodium valproate treatment (Murugesan and Subramanian, 2006), $H_2O_2$-induced hemolysis of human erythrocytes (Sokołowska et al., 1999), or ischemia of the hippocampus (Kovalenko et al., 2011). Based on this, the protective effects of AKG observed here may also be associated with AKG-mediated antioxidant activity, and not only with its well-known cyanide-binding activity. To test this idea that AKG can slow down or quench oxidative stress in SNP-treated flies, we first measured the activity of catalase and levels of oxidative stress markers in 2-day-old females (Table 2), which emerged from pupae raised on a diet with 1 mM SNP or with a mixture of 1 mM SNP and 10 mM AKG (see Fig. 1C). Catalase activity was 57% lower in flies fed with SNP alone, but only 37% lower when food contained the mixture of SNP and AKG, as compared to controls. The decrease in catalase activity can be due to an inactivation of the enzyme as a result of oxidative modification, as previously demonstrated (Bayliak et al., 2006; Semchyshyn and Lozinska, 2012). However, the presence of AKG in food apparently relieved some of this stress, allowing a higher catalase activity to be sustained. Diet composition did not affect the levels of high and low molecular mass thiols in adult females but females reared on SNP showed 1.5-fold higher levels of lipid peroxides (LOOH) than controls, an effect that was abolished when AKG was also present in the diet (Table 2). In addition, nitrite and total iron levels in flies from all experimental groups did not differ (Table 3) in contrast to previous results which demonstrated an increase in these parameters in *D. melanogaster* larvae reared on SNP (Lozinsky et al., 2012). It can be postulated that flies reared on SNP-supplemented food starting from the egg stage may have induced effective mechanisms to metabolize and excrete products of SNP decomposition, thereby minimizing its toxic effects and enhancing survival even they were not fully protected against oxidative damage. Thus, the combined administration of AKG and SNP partially prevented antioxidant enzyme inactivation and oxidative damage of lipids, which agrees with previous studies (Tulsawani et al., 2005; Bhattacharya et al., 2009; Mathangi et al., 2011).

### 3.5. Alpha-ketoglutarate increases resistance of adult flies to hydrogen peroxide

To check whether protective effects of AKG under oxidative stress could be associated with its antioxidant activity, we tested the effect of exogenous AKG on survival of adult Canton S flies exposed to hydrogen peroxide. The latter is commonly used as an inducer of oxidative stress. Flies reared on control and AKG-supplemented food were transferred into vials containing filter paper saturated with 1 M $H_2O_2$ in the presence or absence of AKG at different concentrations. All chemicals were dissolved in 5%

**Table 3**
Total iron and nitrite levels in the bodies of 2-day-old *D. melanogaster* Canton S flies reared on control food or on diets supplemented with SNP or a mixture of SNP and AKG.

| Conditions | Total iron content, μg/mg wet mass | Nitrite level, μg/mg wet mass |
|---|---|---|
| Control | 0.391 ± 0.046 | 0.110 ± 0.013 |
| 1 mM SNP | 0.367 ± 0.041 | 0.104 ± 0.006 |
| 1 mM SNP + 10 mM AKG | 0.342 ± 0.052 | 0.123 ± 0.019 |

Data are presented as the means ± SEM, $n = 4$.

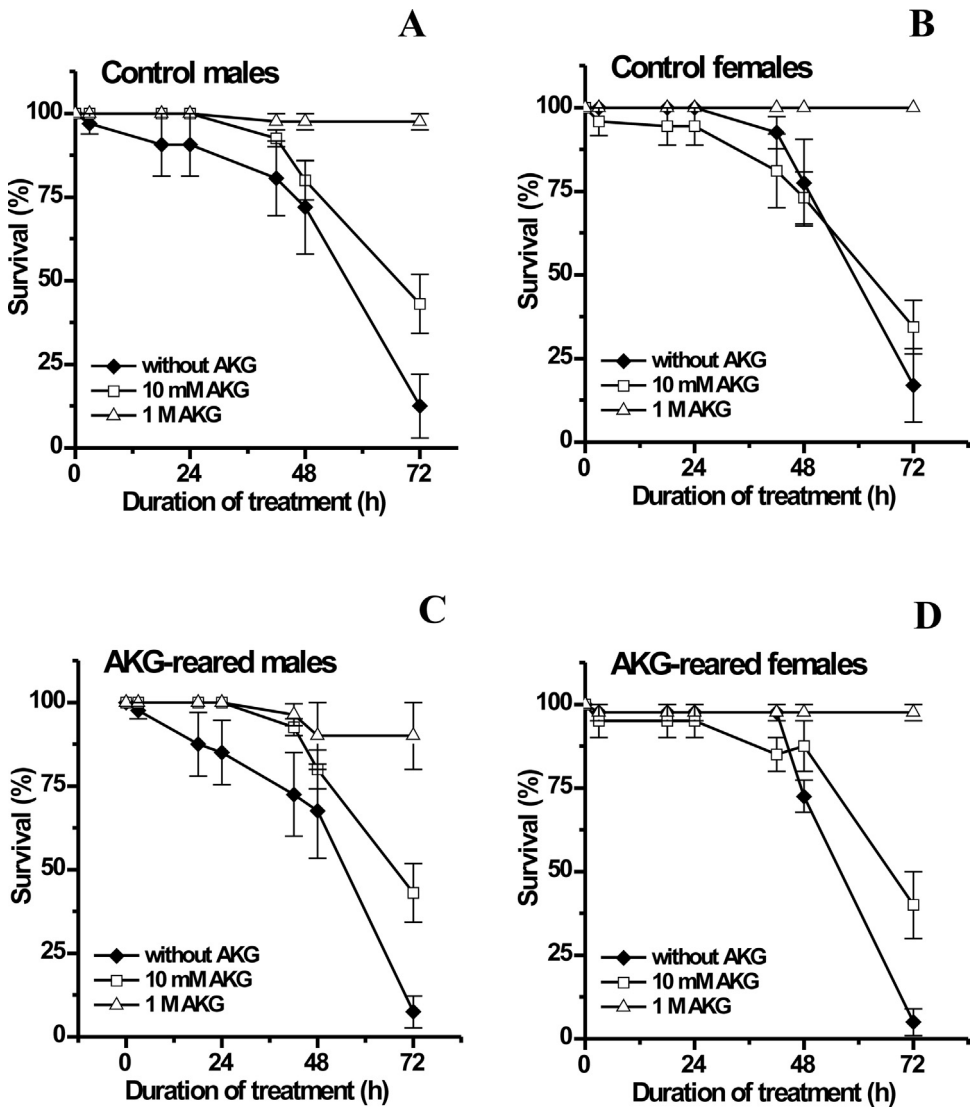


**Fig. 5.** Effect of hydrogen peroxide treatment on the survival of adult *D. melanogaster* Canton S flies. In addition, 2-day-old flies reared on the diet in the absence (control) or presence of 10 mM AKG were divided into three groups which were first starved for 2 h, and then were transferred into new vials containing a filter paper saturated with 0.8 ml of 5% sucrose solution containing either 1 M $H_2O_2$ alone, the mixture of 1 M $H_2O_2$ and 10 mM AKG, or the mixture 1 M $H_2O_2$ and 1 M AKG. Data are presented as the means ± SEM of four independent experiments with 20 flies per sex.

sucrose. The control and AKG-reared flies showed similar sensitivity to 1 M $H_2O_2$ present in the food (Fig. 5). As in the case of SNP treatment, concomitant administration of hydrogen peroxide decreased fly viability over time, although females showed a higher survival for the first 42 h of treatment compared with males. The percentage of flies that survived on $H_2O_2$-treated diets for 42 h was 72.5 and 72% in the case of control and AKG-fed males, respectively, *versus* 92.5 and 97.5% of control and AKG-fed females (Fig. 5). The addition of AKG to the incubation medium alleviated the toxic effects of $H_2O_2$ in a concentration dependent manner. While 10 mM AKG only partly restored fly survival on medium containing 1 M $H_2O_2$, AKG at a concentration of 1 M almost completely neutralized the negative effects of 1 M $H_2O_2$ on fly viability. Upon combined treatment with 1 M $H_2O_2$ and 1 M AKG, 90–100% of flies remained alive after 72 h of incubation in contrast to 5–13% survival when medium contained with 1 M $H_2O_2$ alone or 35–43% survival in the presence of a mixture of 1 M $H_2O_2$ and 10 mM AKG. Several reports have debated the ability of AKG to directly scavenge hydrogen peroxide (Sokołowska et al., 1999; Long and Halliwell, 2011). The beneficial effects of AKG attributed to its antioxidant properties have been previously demonstrated with other model systems both *in vivo* and *in vitro*. It was proposed that at least part of the effect of AKG may be due to a nonenzymatic interaction of AKG with $H_2O_2$ to form succinate, water, and carbon dioxide (Sokołowska et al., 1999). To verify it, we studied the ability of AKG to scavenge $H_2O_2$ *in vitro*. For this experiment, 10 mM $H_2O_2$ was incubated with AKG at different concentrations for 30 min at room temperature and then the levels of AKG and $H_2O_2$ were measured in the solution. The concentration of $H_2O_2$ significantly decreased over incubation time with increasing concentrations of AKG, and $H_2O_2$ was practically absent in solutions incubated with 10 mM AKG for 30 min (Fig. 6). This suggests that AKG can efficiently scavenge $H_2O_2$ *in vitro*. Analyzing the regression equation, we concluded that AKG interacts with $H_2O_2$ stoichiometrically in a molar ratio of 1:1. This can explain the pronounced protective effect of 1 M AKG (*versus* 10 mM AKG) against 1 M $H_2O_2$ (Fig. 5). The protective effect of AKG against hydrogen peroxide toxicity could be based on the chemical interaction in the medium. In addition, this indicates that the antioxidant mode of action of AKG action could also be involved in diminishing the toxic effects of SNP on *D. melanogaster* flies.

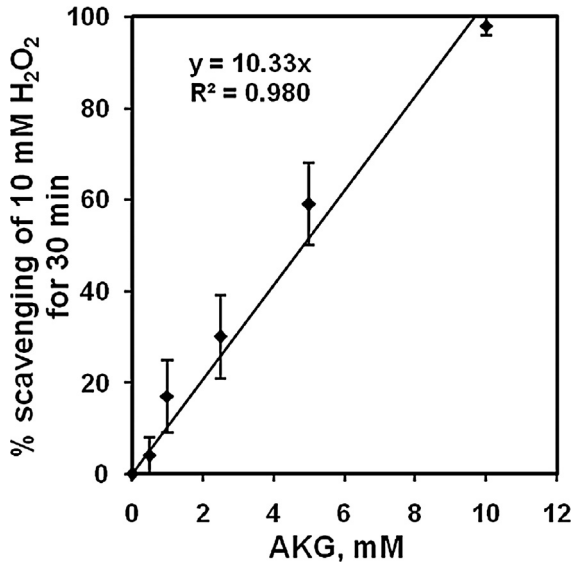


**Fig. 6.** $H_2O_2$ scavenging activity of AKG *in vitro*. Different AKG concentrations were incubated with 10 mM $H_2O_2$ for 30 min and then $H_2O_2$ levels were measured by the FOX method. Data are means ± SEM, $n = 5$.

## 4. Conclusions

The present study demonstrates that supplementation of food with alpha-ketoglutarate partly alleviates the toxic effects of high concentrations of SNP on *D. melanogaster* development, allowing increased numbers of pupae to form and increased pupation rate. In contrast, adult males and females reared on AKG and then exposed to SNP were more sensitive to SNP compared with control flies. This suggests that the AKG consumed may be quickly metabolized and can act as antidote only by combined treatment with SNP. *In vitro* AKG did not affect the rate of SNP decomposition and did not bind iron and nitrite ions released in this process. Exogenous AKG effectively protected adult flies against $H_2O_2$ in combined treatments; this can be explained by its good $H_2O_2$-scavenging activity, which was clearly demonstrated *in vitro*. Dietary AKG also prevented an increase in the levels of oxidative stress markers in SNP-reared adult flies. Thus, it can be concluded that the protective action of AKG against SNP toxicity is realized through both cyanide-binding and antioxidant activities. Our results support the promising utilization of AKG as an antidote against cyanide and cyanide-containing compounds that cause serious problems as industrial pollutants in modern society. In turn, the fruit fly *D. melanogaster* is a useful model for *in vivo* studies of the adverse effects of different toxicants and to analyze potential protective antidotes. Particularly given that cyanide can cause deleterious changes in the nervous system (Mathangi et al., 2011), using antidotes like AKG and *Drosophila* as a model may give insights into which neurobehavioral and biochemical changes appear with age or with long-term poisoning and determine the efficiency of AKG in prevention of these perturbations.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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