Toxicity of Dithiothreitol (DTT) to Drosophila melanogaster

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1. Introduction

Dithiothreitol (DTT or 1,4-Dimercapto-2,3-butandiol) has two SH groups with strong reducibility and is therefore commonly used as an antioxidant in diverse biomedical practices. For instance, DTT prevents interference of the anti-CD38 antibody daratumumab, that is used in a therapy against malignant myeloma, with CD38 exposed on red blood cells during transfusion [1]. Furthermore, DTT has been used in an assay to assess the oxidation property of ambient fine particulate matter 2.5 (PM2.5) in polluted air [2]. Besides these special applications, DTT is a routinely used as a thiol molecule in molecular-biological laboratories. It is applied for protein denaturation.

The biocompatibility of DTT has not been analysed systematically in multicellular organisms and is therefore unclear. In different cell cultures, like other thiols, DTT was reported to induce programmed cell death [3]. There are two proposed mechanisms to explain the effects of DTT in these cases. According to the first possible explanation, thiol oxidation produces hydrogen peroxide following the formula:

$$2\text{RSSH} + \text{O}_2 \rightarrow \text{RSSR} + \text{H}_2\text{O}_2$$

Increased H$_2$O$_2$ levels, in turn, and other reactive oxygen species (ROS) formed in the Fenton reaction ultimately cause lethality [4,5]. A report that catalase, an enzyme that converts H$_2$O$_2$ to H$_2$O and O$_2$, attenuates DTT toxicity to hamster V79 cells supports this hypothesis [4,6]. However, another report suggests that DTT does not promote cell death in HL-60 cells via H$_2$O$_2$ production, but by the activation of caspase 3 [7]. Catalases do not rescue cell death in this case. The second possible explanation is based on the ability of DTT to break disulphide bonds thereby causing protein misfolding [8,9,3]. The accumulation of misfolded proteins, in turn, induces ER stress and ER stress mediated apoptosis. The amplitude of this response depends on the concentration...
of DTT. At a low concentration (3.2 mM), the c-Jun N-terminal kinase (JNK) and p38 are activated upon DTT application. At a high concentration (6.4 mM), in addition, ERK activity is reduced. Treatment of the unicellular parasite Trypanosoma cruzi with DTT interferes with mitochondrial homeostasis, ultimately killing the organism [10]. As T. cruzi does not display a canonical ER stress response, the underlying mechanisms of DTT exposure remain to be studied in detail in this case.

Despite the work on cell cultures, we lack evaluations of DTT toxicity in animals. Here we present our assay of DTT toxicity on the fruit fly Drosophila melanogaster that in a number of cases has served as a model organism in toxicology [11–14].

2. Methods

2.1. Fly husbandry

The D. melanogaster stocks w^{1118} and Dijon 2000 were used in this work. Stocks were kept in vials with standard yeast-cornmeal-molasses food at 25 °C. In all experiments, five to six days old females and males were used. The apoptosis sensor line UAS-GC3Ai was obtained from Magali Suzanne [15]. It was crossed to da-Gal4 for ubiquitous expression in flies of the next generation.

2.2. DTT toxicity assay

DTT (1,4-dithiothreitol, Sigma-Aldrich) stock solution had a concentration of 2% (w/v) in aceton. Dilution of 0.2 %, 0.1 %, 0.02 % and 0.002 % (w/v) were prepared using this stock solution. For each assay, 1 mL DTT solution was applied to a glass vial with a diameter of 1 cm. The concentrations and amounts used were hence 0%-0 mg (aceton alone, negative control), 2 %–20 mg, 0.2 %–2 mg, 0.1 %–1 mg, 0.02 %–0.2 mg, 0.002 %–0.02 mg. Glass vials were placed in a fume hood overnight to allow complete evaporation of aceton. 10 flies were put into the vial that was closed with a cotton plug. The paralysis or knockdown rate as a standard read-out for toxicity in insects [16] was recorded every hour. Knockdown was defined as the inability of coordinated movement and climbing to the top of the vial; these flies eventually died. For analysis of the DTT penetration route, we cut the proboscis with fine scissors one day before the DTT contact assay (Fig. S1). The survived flies were used the next day to evaluate DTT toxicity. To analyse the effect of oral DTT delivery, a feeding assay based on the CAFé assay was designed [17]. 2 % DTT was diluted 1:10 in water and filled into a glass capillary that was inserted into the glass vial to allow the flies to take up DTT water. 0.1 mg/mL chlororanilniliprole and water were used as positive and negative controls, respectively. In this assay, consumption of liquid is visible in the shifting liquid meniscus.

2.3. Quantitative PCR

For quantitative PCR, gene expression in five female flies of each DTT treatment (2 mg, 1 mg, 0.2 mg, 0.02 mg and 0 mg) was analysed. Total RNA was isolated with the RNEasy Kit (Qiagen, Hilden Germany). RNA was used to generate cDNA using oligo(dT) primers (Enhanced Avian First Strand Synthesis Kit, Sigma-Aldrich). qPCR experiments were performed with Roche SybrGreen kit (Roche, Basel, Switzerland) on a Roche LightCycler Nano and evaluated by the respective software. The expression of Rps20 was used as the reference. Four independent samples were prepared in experiments with two technical repeats. Primers for qPCR reaction are listed in Table S1. Student’s T-test was applied for statistical analyses of the expression data.

3. Results and discussion

3.1. DTT is toxic to fruit flies

To characterise DTT toxicity, we coated the bottom of glass vials with four different doses of DTT (20 mg, 2 mg, 0.2 mg, 0.02 mg). At room temperature (25 °C), both male and female flies (w^{1118}, which were expose to the highest DTT amount (20 mg) were all dead after one hour of incubation (Fig. 1). Exposed to 2 mg DTT, most flies survived for several hours, but they lost their ability of coordinated movement and of climbing up the walls of the vial. This effect of paralysis has been reported for a number of insecticides targeting the nervous system, such as DDT and chlororanilniliprole and was called “knockdown” [18]. In vials with 0.2 mg DTT, female flies showed to be more tolerant to DTT than males. With 6 h continuous exposure, only less than 30 % of females displayed the knockdown phenotype, while over 50 % of males were paralysed at the same time. This sexual dimorphic toxin resistance might be due to the different body mass of the genders, females being generally 50 % bigger than males [19]. Flies exposed to 0.02 mg DTT did not show any difference to flies without DTT contact regarding behaviour and mobility. To nevertheless assess any effect of this non-lethal DTT dose on flies, experiments such as larval crawling and climbing assays as described for the evaluation of the toxicity of graphene oxide and zinc oxide nanoparticles on flies shall be performed in the next future [20]. Since the uptake concentration of DTT is unknown, a LC50 value cannot be specified. For the same reason, it is difficult to evaluate the potency of DTT as a toxin. By approximation, compared with DTT, another contact insecticide, the toxic amounts of DTT are ten times higher than the toxic amounts of DDT (0.1 mg) [18]. In an early work, three rats weighing 260 g, 300 g and 300 g, injected with 100 mg DTT died immediately [21]. The molecular consequences of DTT injections were not studied in this case. The companies PanReac Appllichem and Affimetrix (Germany) indicate an LD50 value of 400 mg/kg for rats (oral) and an LC50 (48 h) value of 27.000 μg/l freshwater for Daphnia magna, respectively. Again, no molecular consequences of these tests were reported.

3.2. DTT is more toxic at high temperatures

The efficacy of a toxic substance may be temperature-dependent. This relationship is called temperature coefficient (TC) of the substance toxicity. TCs vary between toxins and insect species and populations. For example, diazinon, pirimicarb, fenazaquin, indoxacar (oxadiazine), teflubenzuron, acetamiprid and beta-cyfluthrin were reported to have positive TCs against the forest beetle Anoplotrupes sterecorosus [22]. Likewise, sensitivity of the brown planthopper Nilapavarta lugens to cycloxaprid, nitenpyram, triflumezopyrim and chlorpyrifos increased with higher temperatures [23]. By contrast, deltamethrin, DDT and pyrethroid were reported to have negative TCs in a number of insect species including the malaria vector Anopheles arabiensis and Anopheles funestus [24,25]. Similarly, nymphs of the bug winchuka (Triatoma infestans) were more resistant to pyrethroids at higher temperatures than at lower temperatures [26].

To further characterize the toxic effect of DTT, we analysed the impact of temperature on DTT sensitivity. In particular, using five doses (Fig. 1), we compared DTT sensitivity at a mild temperature (25 °C) and at a high temperature (33 °C). Without DTT, flies showed normal behaviour and activity at least during six hours of the assay. At the lowest dose (0.02 mg), the flies from both 25 °C and 33 °C cohorts survived during the whole assay. At the highest dose (2 mg), all flies died during the first hour of the assay regardless of the temperature. At the other three doses on average both males and females were more sensitive to DTT at the higher temperature. For example, exposed to 0.1 mg DTT at 33 °C, all females died within 4 h and almost all males died after 6 h. By contrast, at 25 °C, 33 % of the females and 71 % of the males exposed to the same dose of DTT survived after 6 h.

Temperature may affect the efficacy of a toxic substance in different ways. It may have an impact on the stability of the substance, it may influence the interaction between the substance and its target, or it may modulate the metabolic rate of the organism. A cellular mechanism that attenuates TCs is a heat shock response induced by high temperature.
A. arabiensis display higher pyrethroid resistance even after a short-term exposure to high temperature. This induced resistance was demonstrated to be mediated by increased levels of heat shock proteins [25]. In contrast to this linear relationship, some insects developed a trade-off between thermal tolerance and resistance against insecticides. For example, the chlorpyrifos susceptible strain of Plutella xylostella (Diamondback moth) shows higher fitness in terms of longevity and fecundity than resistance strains at high temperatures [27].

In our case, DTT toxicity on D. melanogaster has a positive TC. Trivially, moreover, longer incubation at the higher temperature enhanced lethality of DTT indicating that a possible induction of a heat shock response was unable to attenuate DTT toxicity. One conceivable reason for a positive TC may be the positive correlation between temperature and cuticle permeability to xenobiotics [28, 29]. In our experiments, increased DTT penetration through the cuticle at higher temperatures may explain increased toxicity. Quantification of DTT in the insect body at different temperatures may shed light on this issue. Alternatively, higher metabolic rate at higher temperatures may promote the toxic effect of DTT.

3.3. DTT penetrates the insect body through the cuticle

We next addressed the question as to whether DTT penetrates the insect directly through the cuticle or by ingestion. Two assays were performed to answer this question. First, we removed the proboscis of wild-type flies to make sure that DTT was not taken up by ingestion (Fig. S1). Most of these flies survived to the next day with the wound covered by a melanized scab. They were sensitive to DTT as flies with a functional proboscis (Fig. 2). This suggests that DTT directly penetrates the insect through the cuticle causing the full toxic effect. Second, flies in a vial were watered with DTT resuspended in water via a glass capillary. Uptake of DTT by ingestion did not affect fly activity (Fig. 2). For this assay, as a positive control, the insecticide chlorantraniliprole (0.1 mg/mL) was supplied to fruit flies by the same method. Almost all the flies were knocked down within one hour after insecticide uptake. We conclude that either flies did not ingest a toxic DTT amount in our assay, or that ingestion of DTT is harmless. Together, these experiments indicate that DTT is toxic to D. melanogaster after penetration through the cuticle. Hence, this DTT mode of action corresponds to the mode of action of contact insecticides like DDT [18]. The amounts of DDT that are toxic to flies are, however, ten times lower (0.1 mg). While DDT is a hydrophobic molecule, DTT has two hydroxy groups that may attenuate its penetration through the hydrophobic cuticle. We reckon that the efficient concentration of DTT inside the fly body is, therefore, probably, rather low.

3.4. DTT exposure induced apoptosis

DTT is known to induce apoptosis in cell cultures [3]. We tested whether exposure to DTT may induce apoptosis also in the fruit fly.
Commonly, apoptosis in both mammals and insects are controlled by the stability of caspase inhibitors named the IAPs (inhibitor of apoptosis proteins) [30]. The half-life of IAPs is regulated by IAP-binding-motif (IBM) proteins, which bind to IAPs, elicit IAP degradation and ultimately trigger apoptosis. To some extent, in D. melanogaster, apoptosis is induced by transcriptional upregulation of the genes grim, head-involution defective (hid) and reaper (rpr) that code for Drosophila IAP (DIAP) binding proteins with IBMs [31,32].

Here, we analysed the expression levels of the three IBM protein coding genes in fruit flies after a one-hour exposure to different doses of DTT (Fig. 3A). Although expression levels vary in biological replicates, generally, we observe a dosage-dependent induction of the expression levels of grim, hid and rpr after treatment with different amounts of DTT. Interestingly, the highest induction occurred at the lowest, non-lethal DTT dose (0.02 mg). At this dose, the expression levels of grim increased in the four biological replicates in a range between 5–58 folds compared to the average value of control flies. For hid and rpr, we recorded 1.5–9 folds and 2–30 folds increased expression levels compared to the expression levels in control flies, respectively. At 0.2 mg DTT dose, the grim and rpr expression were also induced by 2–11 folds and 1.5–4 folds compared to control levels, respectively. The hid expression levels of the four biological repeats varied between 0.8 and 16.2 folds of the control average. At 1 mg DTT dose, the expression levels of rpr in the four replicates was higher than the control average levels (1.6–5.9 folds). The expression levels of grim and hid in three replicates were higher and in one replicate lower than control average expression values. At the highest dose (2 mg), the expression levels of the three genes was normal. Based on these data, we assume that at lethal (2 mg) or sublethal (1 mg) doses, flies were unable to mount an apoptosis response because of uncontrolled lethal damages, whereas at lower doses DTT induced a normal apoptosis response. In a next step, we plan to study the cellular survival response in these flies.

We also monitored the expression levels of two IAP coding genes of D. melanogaster, Diap1 and Diap2 (Fig. 3A). Diap1 was identified as the major apoptosis inhibitor [33], while Diap2 was found not to be involved in apoptosis regulation, but in immune response to bacterial infection [34,35]. Usually, the expression of diap2 is constitutive and not regulated by an apoptotic signal. Consistently, the expression levels of Diap1 and Diap2 transcripts were relatively stable in DTT treated flies.

To visualise apoptosis, we expressed the fluorescence apoptosis sensor GC3Ai in the whole fly [15]. Contact with DTT induced fluorescence in the gut tissue, while in control flies no such signal was detected (Fig. 4).

Together, these results suggest that DTT contact induces apoptosis in the fruit fly at the transcriptional level. However, it remains unclear whether apoptosis is the cause of DTT lethality.

3.5. DTT activates the detoxification system at the transcriptional level

Cytochrome P450 monoxygenases (CYPs) and Glutathione S-transferases (GSTs) are two enzyme families that are involved in xenobiotic detoxification in multicellular organisms. CYPs catalyse the reduction of molecules including toxins and insecticides thereby potentially inactivating them [36]. GSTs ligate electrophilic xenobiotic substrates with thiol groups, thereby increasing their solubility and, consequently, their metabolic processing [37]. A number of CYPs and GSTs were found to be implicated in insecticide resistance in different insect species including among many others Spodoptera exigua [38], Anopheles gambiæ [39], Anopheles arabiensis [40], Bombyx mori [41] and Musca domestica [42,43]. In D. melanogaster, several CYP and GST coding genes were expressed at higher levels in a DDT-resistant than in a DDT-susceptible strain [44]. Transgenic overexpression of cyp6g1, cyp6g2 and cyp12d1 in fruit flies conferred selective tolerance against the insecticides DDT (cyp6g1, cyp12d1), nitenpyram (cyp6g1, cyp6g2), dicyclanil (cyp6g1, cyp12d1) and diazinon (cyp6g2) [45]. Additionally, gstD1 was identified as DDTase, which converses DDT to the harmless DDE [46].

CYP and GST expression is also activated upon contact with non-insecticide, but nevertheless toxic xenobiotics. For example, phenobarbital, caffeine or chlorpromazine treatment entails the expression of cyp6g2, cyp6a21, cyp6a8, cyp12d1, cyp6a12, gstD2 and gstD7 in D. melanogaster [47]. Presence of H2O2 was reported to increase the transcript levels of GstD1, GstD2, GstD3, GstD9 and GstE1 in the fruit fly [44].

The detoxification response pathway in insects follows the Cncc/Keap1 signalling pathway, which is also present in mammals [48,49]. In absence of toxic molecules, Cncc (named Nr2f2 in mammals) is present in the cell in a complex with Keap1 and is rapidly degraded by proteasomes. Under oxidative stress, Cncc dissociates from Keap1, enters the nucleus where it associates with Maf and binds to promoters of a number of genes, including CYPs and GSTs to relieve the oxidative stress. To deepen our understanding on the impact of DDT on oxidative stress in D. melanogaster, we plan to analyse the effects of antioxidants on DTT toxicity. A promising antioxidant is curcumin that has been shown to attenuate the adverse effects of copper ions (Cu2+) that induce oxidative stress, for instance, in D. melanogaster and rat renal cells [50,51].

Here, we tested the transcript levels of cncc and six of its target genes including cyp6g1, cyp6a8, cyp12d1, GstD1, GstE1 and GstD2 [47] after DTT exposure (Fig. 3B). Transcript levels of cncc, cyp6g1, GstD1 and GstE1 were unchanged after application of any DTT dose, whereas the

Fig. 3. Topical DTT induces the expression of apoptosis- and detoxification-related genes.
Topical application of low amounts of DTT (0.02 mg) activates the expression of the apoptosis-related genes rpr, grim and hid (A). Diap1 and Diap2 expression, by contrast, is not induced by 0.02 mg DTT. The apoptosis response is less pronounced at higher DTT amounts (0.2 mg–2 mg). A detoxification-response increases with increasing amounts of DTT (B). The expression of the genes oay and sma involved in inward barrier formation is unchanged upon DTT application. Low amounts of DTT (0.02 mg), however, activate the expression of snsl that codes for a barrier forming extracellular protein. Bars indicate standard errors (SE). In cases of seemingly missing bars, the SE values are extremely small.
transcript levels of *cyp6a2*, *cyp6a8* and *GstD2* were enhanced in a dose-dependent manner. These results suggest that DTT induces a detoxification response in the fruit fly body. Regarding the model that DTT produces ROS [3], we speculate that the detoxification response to DTT runs through the Cncc/Keap1 signalling pathway. We note, however, that the signal is selective, activating some of the Cncc targets while some others remain silent. This suggests that despite of being Cncc targets, some genes at the same time are targets of a negative regulator that suppresses their expression in presence of Cncc and DTT. Indeed, a context-dependent response to the Cncc/Keap1 signal has been observed in another example. 70 % of genes, which are upregulated by phenobarbital, are also upregulated in response to Cncc overexpression [47]. On the other hand, only 15 % of Cncc response genes also respond to phenobarbital application. The situation is probably even more complex as there are two additional pathways reported to activate a xenobiotic response in *D. melanogaster*, i.e. the JNK pathway and the DHR96 pathway [52,53]. As the JNK pathway was shown to be responsive to DTT in HeLa cells [3], we tentatively assessed the possibility that it was induced by DTT also in flies by analysing the transcript levels of two JNK targets, namely *atg18b* and *puc* (Fig. 3A). Transcript levels of *atg18b* were unchanged when flies were exposed to DTT; by contrast, *puc* transcripts were highly enriched in DTT-exposed flies. Although the determination of the DTT-induced transcriptome would be necessary for a comprehensive conclusion, we dare to state that only a partial JNK response might be elicited by DTT. We did not test the impact of DTT on the DHR96 pathway as DHR96 is sensitive to starvation to which flies in our assay are exposed to [54]. What we can state, nevertheless, is that transcript levels of *cyp6a8* and *GstD2* were elevated when flies contacted DTT (Fig. 3B), whereas their expression was shown to be independent of DHR96 function [53].

Another genetic program that is activated during general external stress infliction involves the evolutionarily conserved Sestrin protein [55]. Sestrin intervenes with the adenosine monophosphate-dependent protein kinase (AMPK)-Target of rapamycin (Tor) signalling pathway, thereby regulating cellular homeostasis. Among others, Sns function leads to a reduction of ROS amounts. Interestingly, in human cell lines, it has been shown that Sns is able to promote Keap1 degradation, thereby stabilising Nrf2 (the Cncc ortholog) and enhancing its function as a transcriptional regulator [56]. In order to verify whether a Sns-dependent stress response is mounted upon DTT application, we detected its transcripts in DTT treated flies. Indeed, *sens* transcription has been shown to be sensitive to stress [57]. Overall, sens expression was normal in flies exposed to DTT. Thus, Sns is not involved in the DTT-control program in flies.

3.6. Barrier fortification is only induced by non-lethal DTT dosage

The cuticle surface that consists of the envelope and free hydrocarbons (CHCs) is the primary inward barrier to prevent the penetration of xenobiotics [28,29]. In *D. melanogaster*, the ATP-binding cassette (ABC) transporters SnuStorr (Snu) and Oskyddad (Osy) and the extracellular protein SnuStorr-snarlik (Sns) are key proteins involved in the establishment of the surface barrier [58,59]. Snu and Osy belong to the Arthropod-unique ABC transporter subfamily ABCH. Dysfunction of Snu or Osy causes reduction of CHC amounts. Here we monitored the levels of transcripts coding for these three proteins by qPCR following exposure to different amounts of DTT. No change in transcript levels was detected for either of these factors after DTT treatment except for *sns* transcripts at very low DTT amounts compared to the control situation (Fig. 3B). One may speculate that this defence measure is the cause for survival at very low DTT dosage. In summary, we assume that when exposed to very low DTT amounts, flies react with an immediate change in barrier efficiency via alteration of *sns* transcript levels; when exposed to higher DTT amounts, however, rather a detoxification program is launched while barrier fortification is neglected.

**Fig. 4.** DTT induces GC3Ai fluorescence in the gut of adult flies. GC3Ai does not fluoresce in flies that are not exposed to DTT (A). When exposed to DTT (1 mg) in our contact assay, some gut cells show a fluorescence signal indicating that apoptosis has been triggered in them (B). GC3Ai is a GFP version that needs cleavage of DEVD linker in order to emit green light upon excitation with blue light. Images were taken on a Nikon AZ100 microscope.
4. Conclusion

This is the first report on the molecular consequences of DTT toxicity to an animal. Our results indicate that DTT has a toxic effect on D. melanoagaster in a time and dose depended manner. We speculate that DTT may also be toxic to other, especially small animals including vertebrates. Further toxicological investigations are needed to assess the full impact of DTT on ecosystems.

CRediT authorship contribution statement

Yuwen Wang: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. Mailys Misto: Data curation, Formal analysis, Investigation, Methodology. Jing Yang: Data curation, Formal analysis, Investigation, Methodology. Nicole Gehring: Formal analysis, Investigation, Methodology. Xiaoyu Yu: Bernard Moussian: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

This work was supported by a grant from the German Research Foundation (DFG, MO1714/10-1). We thank Magali Suzanne (Université de Strasbourg) for her advice and critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.toxrep.2020.12.014.

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