Physiological and molecular characterisation of cadmium stress in *Schmidtea mediterranea*

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ABSTRACT The planarian *Schmidtea mediterranea* is a well-studied model organism for developmental research, because of its stem cell system. This characteristic also provides a unique opportunity to study stress management and the effect of stress on stem cells. In this study, we characterised the stress signature at different levels of biological organization. The carcinogenic metal cadmium was used as a model chemical stressor. We focused on stem cell activity and its interaction with other known stress parameters. Here, we have found that *S. mediterranea* is able to cope with high internal levels of cadmium. At endpoints such as size and mobility, cadmium-related stress effects were detected but all of these responses were transient. Correspondingly, cadmium exposure led to an elevated mitotic activity of the neoblasts, at the same time points when the other responses disappeared. At the molecular level, we observed redox-related responses that can be linked with both repair as well as proliferation mechanisms. Together, our results suggest that these animals have a high plasticity. The induction of stem cell activity may underlie this ‘restoring’ effect, although a carcinogenic outcome after longer exposure times cannot be excluded.

KEY WORDS: planaria, toxicology, stem cell, cadmium, gene expression

Introduction

Flatworms in general and planarians in particular, are a species-rich taxon of invertebrates and are often abundant in lotic environments (Schockaert et al., 2008), yet little is known about their sensitivity and coping capacity with stress. They are considered to be primitive in certain aspects but meanwhile retained a high degree of morphological plasticity and adaptational capacity. An in vivo accessible pool of stem cells gives them the ability to regenerate themselves (Alvarado, 2004). This is especially useful when studying the effect of carcinogenic compounds. As to cancer cells that do not have the capacity to terminate proliferation, regenerative tissue is able to control and end proliferation (Oviedo and Beane, 2009). Therefore, studying stem cells and their underlying mechanisms under influence of external stressors can provide a better understanding of the basic biology of stress and repair responses.

Free-living flatworms have been used in the past to study the effects of (a)biotic stressors with endpoints on i.e. mortality, regeneration, fecundity, fertility, movement, predation rate, genotoxicity, carcinogenicity, etc. (Kapu and Schaeffer, 1991; Guecheva et al., 2003; Pagan et al., 2006; Knakievicz and Ferreira, 2008; Li, 2008; Kovacevic et al., 2009; Alonso and Camargo, 2011). They are useful in experimental toxicology as they are easy to manipulate in vivo and make it possible to screen for effects at multiple biological levels. As such, toxicity and developmental impairment were evaluated by means of changes in redox homeostasis in the planarian *Dugesia japonica* (Li, 2008). Cell cycle – related parameters such as neoblast mitotic activity, mitotic abnormalities and chromosomal aberrations were measured in *Polycelis felina* to assess the toxicity of cadmium (Kalafatic et al., 2004). It is known that stem cells are involved in both tissue repair responses as well as in cell proliferative and tumorigenic effects. We therefore consider studying stem cells and regeneration of planarians exposed to toxicants as great potential to evaluate underlying mechanisms of repair or

*Abbreviations used in this paper: Cat, catalase; GST, glutathion-S-transferase; GPX, glutathion peroxidase; HSP, heat shock protein; LC, lethal concentration; ROS, reactive oxygen species; SOD, superoxide dismutase.*
carcinogenic processes.

Based on its wide range of effects as well as its carcinogenic properties, cadmium is an ideal stressor to explore this duality and evaluate neoblast activity as a stress parameter. Cadmium is classified as a group 1 carcinogen and because of its threat to the environment and public health, its physiological and biochemical actions are well studied (Nawrot et al., 2010). We designed an experimental set-up that allows us to assess toxicity at different biological levels on one hand, and explore the role of stem cells in stress situations on the other hand. The relation and interaction between these parameters allows us to assess the value of neoblast activity as a toxicological marker, which is discussed within this manuscript.

Results

To determine the concentration range for the following experiments a lethality experiment was conducted. The animals were exposed to varying concentrations of cadmium and mortality was monitored for three weeks. By probit analysis LC10 and LC50 values were calculated (Table 1). At 24 hours the LC50 value was 147.0 μM (confidence interval: 67.2 μM – 371.0 μM CdCl₂; for one week the LC50 value was 39.5 μM (23.1 μM – 66.9 μM CdCl₂ and for three weeks the LC50 value was 30.8 μM (17.1 μM – 61.3 μM CdCl₂). In the following experiments we decided to expose the animals to 2.5, 10 and 25 μM CdCl₂, representing respectively 6.3%, 25.3% and 62.7% of the LC50 value of one week. The results of sublethal exposure experiments were compared with the lethal exposure condition of 100 μM CdCl₂ for which the animals were dead after 48 hours. 100 μM CdCl₂ represents 68.0% of the LC50 value of 24 hours.

We determined whether the dissolved cadmium is actually taken up and accumulated by the animals. Cadmium concentrations were measured after acid digestion, on a whole body basis. The internal cadmium concentration was significantly increased after exposure to 2.5, 10 and 25 μM CdCl₂ after two days and two weeks (Table 2). More specifically, an increase of about 20 times was observed after exposure to 2.5 μM CdCl₂ for 48h and of about 120 times increase after 2 weeks exposure to 25 μM CdCl₂. Higher and longer exposure of cadmium always resulted in higher internal cadmium concentrations.

Planarians have the ability to regulate their body size and change it according to environmental fluctuations (Oviedo and Alvarado, 2003). The body size of worms exposed to 10 μM CdCl₂ for 2 weeks decreased significantly compared to non-exposed worms (Fig. 1). After three weeks, the animals exposed to the lowest cadmium concentrations (2.5 and 5 μM CdCl₂) were shrinking (Fig. 1), exposure to 10 μM CdCl₂ did however not resulted in a significant decrease in body size. Short time exposure for 24 hours to 100 μM CdCl₂ immediately decreased the body surface of the flatworms to 64% ± 4.6% (data not shown).

Behaviour is often used as a parameter to assess overall toxicity. Several systems such as neurological, hormonal and metabolic systems contribute to the performance of normal behavior. The monitoring of mobility is commonly used as a tool to assess the effects on behavior, as this is easy to quantify. Previous studies with flatworms displayed a constant pLMV (planarian locomotor velocity) when tested in water (Raffa et al., 2001). In this experiment a similar protocol was used. In worms exposed to 100 μM CdCl₂ we observed a decline in velocity within the first day of exposure (Fig. 2). On the other hand, worms exposed to 10 μM CdCl₂ displayed an increased velocity from 8 hours to 24 hours. Worms exposed to 2.5 μM CdCl₂ displayed a significant decrease after 48 hours. A significant decrease was also observed in worms exposed to 2.5 and 5 μM CdCl₂ after 72h and to 10 μM CdCl₂ during one week (Fig. 2).

An additional experiment was carried out to investigate if S. mediterranea possesses the ability to avoid cadmium exposure by directing its movements. The animals were monitored during 20 minutes after administering 1 ml of cadmium (32 μM CdCl₂) to the medium on a specific location of the Petri dish. We could not observe any differences in movement directions as compared to

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**Table 1**

<table>
<thead>
<tr>
<th>Concentration (μM CdCl₂)</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
</tr>
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<tbody>
<tr>
<td>LC10</td>
<td>88.5</td>
<td>40.1</td>
<td>30.1</td>
<td>16.4</td>
<td>8.0</td>
<td>16.3</td>
</tr>
<tr>
<td>CI</td>
<td>-39.2–199.2</td>
<td>-27.1–74.3</td>
<td>-72.7–66.2</td>
<td>-11.4–32.7</td>
<td>-26.9–23.8</td>
<td>-5.2–32.0</td>
</tr>
<tr>
<td>LC50</td>
<td>147.0</td>
<td>79.1</td>
<td>76.1</td>
<td>39.5</td>
<td>34.6</td>
<td>30.8</td>
</tr>
<tr>
<td>CI</td>
<td>67.2–371.0</td>
<td>42.3–141.3</td>
<td>34.0–139.1</td>
<td>23.1–66.9</td>
<td>18.3–64.9</td>
<td>17.1–61.3</td>
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</table>

**Table 2**

<table>
<thead>
<tr>
<th>Concentration (μM CdCl₂)</th>
<th>0 μM CdCl₂</th>
<th>2.5 μM CdCl₂</th>
<th>10 μM CdCl₂</th>
<th>25 μM CdCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.27 (0.14-0.80)</td>
<td>0.16 (0.15-0.19)</td>
<td>3.14 (2.55-3.92)</td>
<td>11.19 (9.65-13.53)</td>
</tr>
<tr>
<td>2 days</td>
<td>3.14 (2.55-3.92)</td>
<td>11.19 (9.65-13.53)</td>
<td>23.47 (17.53-30.94)</td>
<td>337.51 (244.36-557.89)</td>
</tr>
</tbody>
</table>
| Estimation (μM CdCl₂) of 10% lethal concentration (LC10) and 50% lethal concentration (LC50) with their 95% confidence interval (CI).

**Fig. 1.** Body area in response to cadmium. The mean and standard error of the body surface of 6 worms exposed to 0, 2.5, 5 or 10 μM CdCl₂ for 1, 2 or 3 weeks relative to the body surface of the control that day. The body surface was expressed as mm². * Significantly (p<0.05) different from non-exposed worms per time point.
Characterisation of cadmium stress in *S. mediterranea*

Neoblasts are the only actively dividing cells in *S. mediterranea*. As such, the basic biology of stem cells can be studied using toxins as tools. New mechanisms that underlie carcinogenesis can be found, and their ability to regenerate allows us to explore anti-cancer strategies when these cells are coping with carcinogens or other stressors. Staining of the dividing cells in this species allows the localization and quantification of active and dividing neoblasts. To investigate the number of neoblasts divisions we used a mitotic marker that recognizes Histone H3, when it is phosphorylated at serine 10 (anti-H3P), following cadmium exposure. The neoblast cell proliferation was significantly elevated by the exposure to 10 μM CdCl$_2$ for 2 weeks (p < 0.05) (Fig. 3). Increased mitotic division was still observed after 3 weeks exposure to 2.5 and 5 μM CdCl$_2$ (p<0.05). The presence of cadmium-related effects was studied on the ultrastructural level in the epidermis and undifferentiated neoblasts of animals exposed to 10 μM CdCl$_2$ for 1, 2 and 3 weeks. Based on the results of Braeckman et al., (1999) following cadmium-related effects were studied: nuclear chromatin clumping, indentation, filling and dilatation of the perinuclear cisternae, condensation and/or dilatation of mitochondria, the presence of increased amounts of free and membrane bound ribosomes, filling and dilatation of the rough endoplasmic reticulum and an increase of the lysosomal system. However, no significant effects were observed on the ultrastructure of neither the neoblasts nor the epidermis (data not shown).

**Fig. 2. Mobility in response to cadmium.** Box plot of the fraction of lines crossed in 4 minutes by cadmium-exposed groups (2.5, 5, 10 and 100 μM CdCl$_2$) compared to non-exposed worms for (A) 0.5, 1, 4, 8 and (B) 24, 48 and 72 h, and 1, 2 and 3 weeks. The bold line indicates the mean and the thin line indicates the median. The closed circles stand for the 5th and 95th percentile. * Significantly (p<0.05) different from non-exposed worms per time point. The table (C) provides mean and standard errors for the non-exposed worms.

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean ± se</th>
<th>Time</th>
<th>Mean ± se</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>16.55±1.41</td>
<td>48h</td>
<td>19.5±0.83</td>
</tr>
<tr>
<td>0.5h</td>
<td>17.88±1.13</td>
<td>72h</td>
<td>17.79±1.70</td>
</tr>
<tr>
<td>1h</td>
<td>17.22±2.72</td>
<td>1 week</td>
<td>12.67±2.92</td>
</tr>
<tr>
<td>4h</td>
<td>14.14±2.32</td>
<td>2 weeks</td>
<td>18.36±1.40</td>
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<tr>
<td>8h</td>
<td>15.41±2.53</td>
<td>3 weeks</td>
<td>12.81±1.75</td>
</tr>
<tr>
<td>24h</td>
<td>17.09±2.41</td>
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</table>
Cadmium is known to indirectly cause oxidative stress. Organisms have developed a powerful antioxidant defense system to minimize or prevent deleterious effects from ROS exposure. To study the balance of oxidative stress we measured the enzymatic activity of the enzymes involved in the antioxidative defence (Table 3). Compared to the control situation, a significant reduction of catalase (CAT) activity was noticed after 3 days of exposure to 5 and 10 μM CdCl₂ and after 1 week of exposure to 10 μM CdCl₂. Both glutathione-S-transferase (GST) and superoxide dismutase (SOD) showed significant increases as well as decreases in enzymatic activity depending on the exposure condition. SOD activity was inhibited at the lowest exposure levels (2.5 and 5 μM CdCl₂) during the first days of exposure. An exposure of 10 μM CdCl₂ during 3 days and an exposure of 5 μM CdCl₂ during 1 week resulted in significant increases of SOD activity. In case of GST activity, an early induction after 1-day exposure was followed by a decrease at later time points.

Gene expression changes associated with signal pathway activation can provide compound-specific information on the pharmacological or toxicological effects of toxicants. Hence, we observed the effects of cadmium on gene expression patterns of heat shock proteins (hsp60, hsp70, mapk38, p53 and anti-oxidative enzymes (gpx and sod). After exposure to CdCl₂ an early inhibition on hsp60 and hsp70 was detected (Table 4). However, after 2 days an increase in hsp70 was observed (Table 4). Exposure to 100 μM CdCl₂ significantly increased (p < 0.05) the hsp70 gene expression with 3.88 (standard error: 0.31) times after 4 hours, and 10.68 (standard error: 2.56) times after 24 hours (data not shown). Mapk38 and p53 gene expression were also decreased as an early reaction to CdCl₂ exposure. After the initial decrease, p53 transcripts were elevated, especially after 2 weeks exposure to cadmium. Mapk38 was upregulated after 2 days. Gpx expression was decreased both during the early hours of cadmium exposure as well as after 1 and 2 weeks exposure to 2.5 and 5 μM CdCl₂ (Table 4).

**Discussion**

Planarians offer the advantage of studying stem cells in an *in vivo* situation. From a toxicological point of view, stem cell dynamics can be followed in function of a specific stress situation (Kalafatic et al., 2004; Knakievicz et al., 2008). This is especially interesting when studying the effect of carcinogenic compounds, as stem cells are involved in both tissue repair responses as well as in cell proliferative and tumorigenic effects.

In this study, we used the metal cadmium as a chemical stressor to evaluate stem cell activity in relation to other stressors. The planarian *Schmidtea mediterranea* was used as a model organism and toxicity was assessed on basic macroscopic toxicity and mortality, as well as on metabolic, transcriptomic and ultrastructural level. The usefulness of stem cell activity as a new toxicological parameter is discussed throughout the manuscript.

**Overall sensitivity to cadmium**

To assess the overall sensitivity level of *S. mediterranea* to cadmium stress, we compared its acute toxicity data with a distribution of species sensitivity values determined from an analysis of existing cadmium toxicity data from various genera of fresh water organ-

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**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>1 Day</th>
<th>2 Days</th>
<th>3 Days</th>
<th>1 Week</th>
<th>2 Weeks</th>
<th>3 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 μM CdCl₂</td>
<td>5 μM CdCl₂</td>
<td>10 μM CdCl₂</td>
<td>2.5 μM CdCl₂</td>
<td>5 μM CdCl₂</td>
<td>10 μM CdCl₂</td>
</tr>
<tr>
<td>CAT</td>
<td>1.23 ±0.12</td>
<td>1.26 ±0.37</td>
<td>1.03 ±0.36</td>
<td>0.51 ±0.34</td>
<td>0.95 ±0.35</td>
<td>1.16 ±0.35</td>
</tr>
<tr>
<td>GPX</td>
<td>0.34 ±0.23</td>
<td>0.65 ±0.30</td>
<td>0.29 ±0.18</td>
<td>1.51 ±0.76</td>
<td>1.87 ±0.62</td>
<td>1.01 ±0.29</td>
</tr>
<tr>
<td>GST</td>
<td>1.13 ±0.93</td>
<td>2.43 ±0.65</td>
<td>0.89 ±0.55</td>
<td>1.33 ±0.56</td>
<td>0.75 ±0.56</td>
<td>0.28 ±0.43</td>
</tr>
<tr>
<td>SOD</td>
<td>0.72 ±0.28</td>
<td>1.26 ±0.51</td>
<td>0.51 ±0.07</td>
<td>0.29 ±0.20</td>
<td>0.29 ±0.15</td>
<td>0.72 ±0.73</td>
</tr>
</tbody>
</table>

Worms were exposed to 2.5; 5; 10 μM CdCl₂. Values are mean±s.e. of six independent biological replicates, relative to control group. Nd = not determined.

* Significantly increased or decreased as compared to the non-exposed animals (p-value < 0.05).
Cadmium accumulates in the body in relation to exposure conditions

Non-lethal endpoints are more sensitive than mortality, can be used as indicators of early toxicity and provide information on the mode of action. The internal dose of cadmium is significantly and strongly elevated in function of exposure time and concentration (Table 2). Although these animals are rather insensitive to cadmium at the level of mortality, an increased accumulation without obvious toxic effects may suggest a high plasticity. The difference in increase of cadmium body burden between 2 days and 2 weeks was not linear suggesting an induction of accumulation rate in function of time.

The cadmium body burden in this study was within the same range as the internal cadmium concentration found in the flatworm Dugesia japonica (Wu et al., 2011). These researchers showed a higher cadmium accumulation in the head of planarians, associated with an increased level of metallothioneins, which are known for their metal binding and regulating capacity.

Size and behaviour to assess overall toxicity

Sublethal responses such as behaviour and size are used in different invertebrate studies to evaluate the effects of contaminants. Negative effects of cadmium on growth rate have been demonstrated for chironomids (Postma and Davids, 1995), for Daphnia magna (Biesinger and Christensen, 1972) and for soil arthropods (Janssen and Bedaux, 1989). Our data also shows a decrease in body size, but the animals seem to be able to recover as the body size of the worms exposed to 10 μM CdCl₂ returned back to the control level (Fig. 1). Planarians are known to change in body size depending upon whether they are in feeding or starving conditions (Oviedo and Alvarado, 2003). The observed decrease can be either a stress effect or the result of muscle contraction as part of the defence strategy. Anyhow the worms are able to cope with the increasing internal cadmium level, possibly as a result of the increased cell proliferation (Fig. 3). Based on the cell proliferation data, we hypothesise that the worms exposed to 5 μM CdCl₂ will also restore their body size after a longer exposure period (Fig. 3).

Our results indicate that behaviour is a relatively sensitive parameter during low-level exposure. Significant changes are already detected after an exposure time of 8 h, 24 h, 48 h and 72 h. This is in accordance with the study by Zhang et al., 2010 where a decrease in behavioural activity in planarians exposed to subtoxic concentrations of cadmium was reported. Behavior can be used to assess the effects on neurological and developmental processes (Eriksson, 1997) The follow-up of this parameter also indicates a coping strategy and recovery was observed after two and three weeks of exposure.

Toxicodynamics at cellular level

The neoblast activity is triggered by the elevated internal cadmium concentrations. Both parameters increase in function of exposure time and concentration (Fig. 3), which is in contrast with the cadmium-induced inhibition of the neoblast mitotic activity in Polyceps felinea (Daly) (Kalafatic et al., 2004). Cadmium is a potent carcinogen and any disturbance of the balance between cell proliferation, differentiation and apoptosis can contribute to the cancer process. On the other hand, neoblasts are the only dividing cells in an adult organism and hence the only source for tissue maintenance and cell renewal (Reddien and Alvarado, 2004). The proliferation can be a defence strategy to eliminate or store the accumulated cadmium or repair lost tissues. This high degree of plasticity can be responsible for the absence of tissue damage at the ultrastructural level. It is however not clear if this increased cell proliferation eventually can lead to the development of a tumor. Based on their ultrastructure, we do see that neoblasts are resistant when exposed to sublethal concentrations, as has been previously established in human embryonic stem cells (Saretzki et al., 2004). This may be the result of one major pressure exerted; i.e. the need to maintain stemness (Prinsloo et al., 2009). The potential higher telom-

### Table 4

TRANSCRIPT LEVELS OF HSP60, HSP70, MAPK P38, P53, GPX AND SOD OF WORMS EXPOSED TO 2.5, 5, 10 μM CdCl₂, EXPRESSED RELATIVE TO THE CONTROL GROUP

<table>
<thead>
<tr>
<th></th>
<th>2.5 μM CdCl₂</th>
<th>5 μM CdCl₂</th>
<th>10 μM CdCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 60</td>
<td>0.77 ± 0.01</td>
<td>0.63 ± 0.02</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>Hps 70</td>
<td>0.26 ± 0.02</td>
<td>0.46 ± 0.09</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>M p38</td>
<td>0.32 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>P53</td>
<td>0.65 ± 0.03</td>
<td>0.39 ± 0.02</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>Gpx</td>
<td>0.60 ± 0.03</td>
<td>0.48 ± 0.02</td>
<td>0.39 ± 0.01</td>
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<tr>
<td>Sod</td>
<td>0.97 ± 0.05</td>
<td>1.09 ± 0.03</td>
<td>2.16 ± 0.04</td>
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</table>

Values are means of six independent biological replicates. ■ Significant decreased/increased as compared to the non-exposed animals (p-value < 0.05).
erase activity of stem cells can protect these cells to the effects of oxidative stress, one of the most important cadmium-induced effects (Yang et al., 2008). As a trigger of both (uncontrolled) cell proliferation and repair, the redox status of the organisms was evaluated to further elucidate this ambiguity (Cuypers et al., 2010).

**Toxicodynamics at molecular level**

Responses to oxidative stress in animals involve the activation of common enzymes such as catalase, superoxide-dismutase, etc. By measuring their activity the ROS removing capacity of the worms is determined. Fluctuations in antioxidant capacity indicate disturbances in the redox homeostasis, a situation that is called oxidative stress. Oxidative stress is visible in our data as considerable changes in SOD, CAT and GST activities were detected during the exposure period (Table 3). Their capacity to detoxify newly formed ROS is varying over the course of the experiment. No permanent changes were observed after the longest exposure period, again indicating a high coping level.

In contrast to the enzyme activity of GPX and SOD, we could not establish significant changes in the gene expression of sod but on the other hand did find significant changes in the gene expression of gpx. Lack of correlation among gene expression and enzyme activity of catalase and other antioxidant enzymes has also previously been reported (Kim et al., 2010). This discrepancy might be due to the fact that enzyme activities are also modified post-transcriptionally (Hansen et al., 2007), the half-life of mRNA and proteins varies (Taniguchi et al., 2010) or multiple isoforms of the studied molecules exist.

The upregulation of hsp genes is another indication for oxidative stress correlating to the diminished anti-oxidative capacity of SOD, GST and CAT (Table 3). Hsp60 and hsp70 showed an initial inhibition as an early response to cadmium, after which their expression increased and returned to the control level (Table 4). Despite the fact that Hsp expression was not persistently upregulated in our study, we hypothesise that HSPs are important in the neoblast defence, guiding survival and upregulation during cadmium stress. In Dugesia japonica, a member of the HSP70 family, mortalin is essential for neoblast viability and regeneration in the planarian Dugesia japonica (Conte et al., 2009). However, the stress-induced upregulation of the hsp60 transcript was not indispensable for tissue regeneration (Conte et al., 2010).

The control of cell proliferation has also been attributed to a mechanism of Mortalin-dependent cytoplasmic sequestration of the p53 tumour suppressor protein (Wadhwa et al., 2002). It is possible that the observed activation of p53 (Table 4) is counteracted by an induced HSP response, indicating the hypothesis that heat shock proteins via p53 are involved in the cadmium-induced stem cell proliferation. Nevertheless, more detailed knowledge about all HSP molecules is required to verify this theory.

**Conclusion**

From a fundamental perspective planarians provide unique opportunities to study stress management and the effect of stress on stem cells. In this study we found that S. mediterranea is able to cope with high internal levels of cadmium. At all studied biological levels, cadmium-related stress effects were detected but none of these responses were permanent. We hypothesise the 2 and 3 week – related induction of stem cell activity is underlying to this ‘restoring’ effect, although a carcinogenic outcome after longer exposure times can not be excluded.

**Materials and Methods**

**Test organism**

Asexual strains of the freshwater flatworm Schmidtea mediterranea (Baguña, 1973; Benazzi et al., 1975) were maintained in the dark at room temperature (20 °C) in water, that is deionised then distilled, with 1.6 mM NaCl, 1.0 mM CaCl2, 1.0 mM MgSO4, 0.1 mM MgCl2, 0.1 mM KCl and 1.2 mM NaHCO3. In order to generate genetically identical animals, strains were created by serially amputating individual worms followed by regeneration of these fragments. The animals were fed once a week with beef liver.

During the experiments the animals were exposed to cadmium chloride (CdCl2·H2O) in Petri dishes with 20 ml of medium and fed once a week, the medium was refreshed twice a week. Worms that fissioned were excluded from the experiment.

**Lethality experiment**

Nine different cadmium concentrations (0, 5, 10, 20, 50, 70, 90, 100 and 150 μM CdCl2) at 6 time points (24h, 48h, 72h, 1 week, 2 weeks, 3 weeks) were used for the lethality test. The experimental setup was blinded and randomized and 10 worms were used per condition. The status of the animals (dead or alive) was controlled on the different time points scoring 0 for dead animals and 1 for living animals.

The concentration for which respectively 10% or 50% of the animals died, the LC10 and LC50 values were calculated using a probit-analysis, with SAS9.2. The lethality experiment was replicated three times.

**Cadmium accumulation**

Worms were exposed to 0; 2.5; 10 and 25 μM CdCl2 for 2 or 21 days. At sample collection, the organisms were washed twice and put in a fresh Petri dish with water that is first deionised then distilled. Ten worms per sample were placed on a piece of aluminium foil (5x5 mm), as carrier of the worms, with a toothpick that was dipped in liquid nitrogen. The carriers were collected in 6 well plates and dried for 48 h at 60 °C. The mass of the dried worms was determined with a microbalance (0.001 mg). The worms were dissolved in nitric acid in a heat block and the digests were diluted with ultrapure water. The sample was measured on an ICP-MS (inductively coupled plasma – mass spectrometry). The concentration of cadmium was by means of a standard curve determined. The results were expressed on a dry weight basis.

**Body area**

Worms were exposed to 0; 2.5; 5 and 10 μM CdCl2 and their size was determined at different time points (1 week, 2 weeks, 3 weeks). In addition to this experiment worms were exposed to one highly toxic concentration of 100 μM CdCl2 for 24 hours. To determine the size of the animals the worms were individually placed in a Petri dish and photographed using a digital camera (DFK 41AF02 FC ccd camera ( Imaging Source)) and a stereomicroscope (Nikon). The surface area was measured on the digital image using a Java image processing program Image J from the National Institute of Mental Health (USA).

**Mobility**

The mobility test was modified from the locomotion activity setup of Raffa et al., (2001). The worms were exposed to 0; 2.5; 5; 10 and 100 μM CdCl2 and their locomotion velocity was recorded at different time points (0.5h, 1h, 4h, 8h, 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks). The experimental setup was blinded and randomized. At each time point individual animals were placed in a Petri dish with medium or cadmium solutions above a grid (squares of 0.5 cm²). A cold light source was placed 18 cm above the animals. After a rest period of 4 minutes the number of lines crossed per minute was scored for 4 consecutive minutes.

To test if adding cadmium to the medium provoked escaping or evok-
ing behaviour an adjusted protocol modified from Wisenden and Millard (2001) was used. One worm was placed in a Petri dish containing 30 ml medium above a grid with squares of 0.5 cm², ranked according to risk area, generating a high score near the place of injection and a low score distant from the injection. A cold light source was placed 18 cm above the animals. After a rest period of 4 minutes, either 1 ml of cadmium solution (32 μM CdCl₂) or 1 ml of the medium was during 30 seconds injected in the medium of the Petri dish containing the worm. This experiment was performed with 25 worms for the cadmium as well as with 25 other worms for the control injections. Every test was performed in a new Petri dish. The position of the worms was observed every 10 seconds by means of student t-test statistics.

Immunohistochemistry

The worms were treated with five–eighths Holtfreter solution containing 2% HCl for 5 minutes on ice to remove the mucus layer. The samples were fixed in Camoy’s fixative for 3 hours on ice and were rinsed in 100% methanol during 1 hour and bleached overnight at room temperature in 6% H₂O₂ in methanol. Subsequently, the worms were rehydrated through a graded series of methanol/PBST washes (75%, 50%, 25%) for 10 minutes each, in methanol. Subsequently, the worms were rehydrated from methanol during 1 hour and bleached overnight at room temperature in 6% H₂O₂.

The fixed tissues were rinsed 2 times 20 minutes in 0.1 M X-100, and 0.1 mg/ml BSA) for 3 hours. Animals were incubated at 4 °C in 2.5 % glutaraldehyde buffered in 0.1 M sodium cacodylate. Subsequently, the tissues were fixed in Carnoy’s fixative for 3 hours on ice and were rinsed in 100% methanol. The fixed tissues were rinsed 6 times in PBST and mounted in glycerol.

The animals were examined with fluorescence microscopy performed with a Nikon Eclipse 80i, equipped with and a ccd camera (DFK 41AF02 FC, Imaging Source). The total number of neoblasts was normalised to the body size of the animals (cfr. Body area), determined by measuring the surface of the fixedated animals in Image J.

Ultrastructure

Tissue samples (max 1 mm³) from S. mediterranea were fixed for 4 hours at 4 °C in 2.5 % glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.3). The fixed tissues were rinsed 2 times 20 minutes in 0.1 M sodium cacodylate and post-fixed for 1 hour at 4 °C in 2 % osmium tetroxide, buffered in 0.05 M sodium cacodylate. Subsequently, the tissues were rinsed 2 times 30 minutes in 0.1 M sodium cacodylate. After dehydration in a graded acetone series, the tissues were impregnated and embedded in Spurr’s epoxy resin. Ultrathin sections (65 nm) were obtained using a Leica Ultracut UCT ultramicrotome and mounted on coated copper grids (50 mesh).

The sections were examined using a Philips EM 208S transmission electron microscope operating at 80 kV and digitized with a Morada 3.0 TEM camera controlled by iTEM FEI (version 5.0) software from Olympus Soft Imaging Solutions GmbH.

Enzymatic activity

Frozen worms were mixed (Mixer mill, MM 2000, Retsch) in 1 ml ice-cold 0.1 M Tris–HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 4% insoluble polyvinylpyrrolidone. The homogenate was centrifuged for 12 min at 1200rpm (MR22i Jouan) and 4 °C. The enzyme activities were measured spectrophotometrically (UV-1602, Shimadzu) in the supernatant at 20 °C. The enzyme activities were normalised to the size of the animals (cfr. body area)

Catalase activity was measured at 240 nm according to Bergmeyer et al. (1974). Superoxide dismutase was determined by measuring the NBT-diformazan decrease at 560 nm according to Beauchamp and Fridovich (1971). Gluthatione peroxidase was measured at 340 nm by calculating the change in NADPH. Analysis of glutation-S-tranferase activity was based on the change of S-2,4-dinitrofenylglutathion at 340 nm.

Gene expression

The worms were exposed to 0; 2.5; 5 and 10 μM CdCl₂ and 5 samples per condition were snap frozen at different time points (4h, 8h, 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks). For hsp70 gene expression, additional, extra samples were generated as the animals were also exposed to 0 and 100 μM CdCl₂ for 4 and 24 hours. Frozen animals were disrupted under frozen conditions using a Retsch Mill MM2000 equipped with a single stainless steel bead of 2 mm diameter. The mechanical disruption was followed by chemical lysis in 200 μl RNA lysis/binding buffer (Qiagen, Venlo, The Netherlands) including 1% β-mercaptoethanol. RNA was isolated using a phenol-chloroform extraction procedure (Chomczynski et al., 2006), and was precipitated with Na-acetate and 70% ethanol. RNAconcentrations were assessed on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and all RNA samples were adjusted to 200ng, measured and re-adjusted again to homogenize RNA input in the subsequent cDNA synthesis reaction. RNA quality was checked using an Agilent-2100 Bioanalyzer and RNA 6000 NanoChips (Agilent Technologies). Genomic DNA was removed with the Turbo DNA free kit (Ambion). 200 ng RNA resolved in 12 μl, 1.5 μl Turbo DNase buffer and 1 μl Turbo DNase (1/4 diluted) was added, then these reagents were incubated for 30 minutes at 37 °C. Two μl DNase inactivation reagent was added. Ten μl of this mixture was used for reverse transcription with the High capacity cDNA reverse transcription kit with RNAse inhibitor (Applied Biosystems) following manufacturer instructions. The cDNA was 1/10 diluted in 1/10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, 0.05% NaN₃).

<table>
<thead>
<tr>
<th>TABLE 5</th>
<th>REAL TIME PCR PRIMERS</th>
</tr>
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<tbody>
<tr>
<td><strong>Actin</strong></td>
<td>act</td>
</tr>
<tr>
<td><strong>beta-Tubulin</strong></td>
<td>tubb</td>
</tr>
<tr>
<td><strong>Cytochrome C oxidase subunit IV</strong></td>
<td>cox4</td>
</tr>
<tr>
<td><strong>Casein kinase 2</strong></td>
<td>ck2</td>
</tr>
<tr>
<td><strong>Cystatin</strong></td>
<td>cys</td>
</tr>
<tr>
<td><strong>Glycolaldehyde 3-phosphate dehydrogenase</strong></td>
<td>gapdh</td>
</tr>
<tr>
<td><strong>GM2 ganglioside activator</strong></td>
<td>gm2a</td>
</tr>
<tr>
<td><strong>Phospholipid scramblase 1</strong></td>
<td>plsc1</td>
</tr>
<tr>
<td><strong>Ribosomal protein L13</strong></td>
<td>rpl13</td>
</tr>
<tr>
<td><strong>Heat shock protein 70</strong></td>
<td>hsp70</td>
</tr>
<tr>
<td><strong>Heat shock protein 60</strong></td>
<td>hsp60</td>
</tr>
<tr>
<td><strong>PS3</strong></td>
<td>ps3</td>
</tr>
<tr>
<td><strong>Glutathion peroxidase</strong></td>
<td>gpx</td>
</tr>
<tr>
<td><strong>Superoxide dismutase Copper/Zinc</strong></td>
<td>sod</td>
</tr>
<tr>
<td><strong>Mitogen activated protein kinase p38</strong></td>
<td>mapk38</td>
</tr>
</tbody>
</table>
0.1 mM EDTA, pH 8.0) before storage at -20°C.

Primer sequences of the reference genes were determined from the S. mediterranea genome database (Robb et al., 2008), see Table 5. Real-Time PCR was performed in an optical 96-well plate using a ABI PRISM 7000 sequence detection system (Applied Biosystems) under universal cycling conditions (10 min 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). Subsequently a dissociation curve was generated in order to check for specificity of amplification. Reactions contained SYBR Green Master Mix (Applied Biosystems), 0.3 μM of a gene-specific forward and reverse primer, and 2.5 μl of the diluted cDNA in each 10 μl reaction. “No template controls” contained 2.5 μl RNase-free water instead of cDNA. Quantification cycles were automatically determined by the software of the ABI PRISM 7500. Primer efficiencies were calculated as $E = 10^{-ΔΔCt}$ on a standard curve, and efficiencies of 0.85 to 1.15 were tolerated. Reference genes were selected according to geNorm analysis (adjusted from Plusquin et al., 2012). Gene expression data were calculated relative to the reference gene following the 2$^{-ΔΔCt}$ method (Livak and Schmittgen, 2001). Gene expression was performed with MIQE guidelines taken into account (Bustin et al., 2009).

Based on the results of the Shaprio-Wilk test for normality statistical significance of differences between means was determined by means of ANOVA or non-parametric Krustal-Wallis test. $P$-values less than 0.05 were considered significant.

**Statistics**

Unless above described for specific experiments, statistical significance of differences between means was determined by means of ANOVA or for non-parametric data the Krustal-Wallis test. $P$-values less than 0.05 were considered significant. The statistical analyses were performed using SAS 9.2 and Excel.

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