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The *in vivo* effect of chelidonine on the stem cell system of planarians

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Abstract

The presence of adult pluripotent stem cells and the amazing regenerative capabilities make planarian flatworms an extraordinary experimental model to assess *in vivo* the effects of substances of both natural and synthetic origin on stem cell dynamics. This study focuses on the effects of chelidonine, an alkaloid obtained from *Chelidonium majus*. The expression levels of molecular markers specific for stem or differentiated cells were compared in chelidonine-treated and control planarians. The use of these markers demonstrates that chelidonine produces *in vivo* a significant anti-proliferative effect on planarian stem cells in a dose-dependent fashion. In response to chelidonine treatment mitotic abnormalities were also observed and the number of cells able to proceed to anaphase/telophase appeared significantly reduced with respect to the controls. Our results support the possibility that chelidonine acts on cell cycle progression by inhibition of tubulin polymerization. These studies provide a basis for preclinical evaluation *in vivo* of the effects of chelidonine on physiologically proliferating stem cells.

Keywords: Chelidonine, Planarian, Stem Cells, Cell proliferation, Molecular Markers

1. Introduction

Stem cells are, by definition, undifferentiated cells that have both the ability to self-renew, making identical copies at each division, and to differentiate specialized cells. These cells offer the possibility of a renewable source of replacement cells and tissues, with potential applications in regenerative medicine. However, a number of pathological conditions, such as cancer and psoriasis, are characterized by hyper-proliferative phenomena. Consequently, inhibition of cell proliferation results to be potentially useful in drug discovery to design agents targeted to the treatment of diseases characterized by uncontrolled or abnormal cellular proliferation. In this context, the so-called adult or somatic stem cells (ASCs) have attracted special attention. *In vivo* studies of ASCs are not easy in mammals due to the difficulty of developmental biology studies *in vivo* using vertebrate models. For this reason, most of the knowledge on ASC biology results from *in vitro* studies. Non-mammalian model systems can be alternatively used to assess *in vivo* the effect of substances on these cells.

Planarians are an ideal animal model for *in vivo* analysis of ASCs. Their extraordinary ability to regenerate, as well as to sustain continuous homeostatic turnover of all differentiated cell types, relies on the presence of stem cells that share characteristics with the stem cells of higher organisms, including humans. Planarian regeneration involves remodeling of pre-existing tissues and formation of an outgrowth at wound region, in which the missing parts are newly formed (the regenerative blastema) (Gentile et al., 2011; Slack, 2011). The discovery that planarian stem cells use evolutionarily conserved mechanisms that are surprisingly similar to the mechanisms we observe in mammalian stem cells, makes these invertebrates suitable to screen the effects of new drugs on stem

cells. In addition, the planarian genome shares a large number of genes with the human genome, including sequences involved in various aspects of human biology and disease. Our work focuses on the analysis of the effects produced *in vivo* by substances of natural origin, particularly a series of compounds contained in *Chelidonium majus*, on planarian stem cells. *C. majus* (greater celandine) is a herbaceous plant of the *Papaveraceae* family that spontaneously grows in temperate areas. As a starting point we studied the effects of chelidonine, an isoquinoline alkaloid derived from *C. majus*. The pharmacological potential of mitotic arrest by chelidonine is considered promising in cancer therapy. In this paper the activity of chelidonine was tested *in vivo* in *Dugesia japonica*, a clonal planarian strain widely used to study drug responsiveness (Kitamura et al., 2003; Nogi and Levin, 2005; Nogi et al., 2009).

2. Materials and methods

2.1. Animals and treatments

Asexual specimens of *D. japonica* (GI strain) were maintained at 18°C in autoclaved stream water, fed weekly with chicken liver and starved one week before the experiments. Control groups were represented by planarians soaked in dimethylsulfoxide (DMSO) at different concentrations in the absence of drug and by untreated planarians, maintained in water. As literature data demonstrate that DMSO concentrations >0.1% should be avoided in order to be able to reliably observe any behavioral or toxic effects of hydrophobic drugs in these animals (Pagán et al., 2006; Yuan et al., 2011), we first analyzed the long-term effects of DMSO exposure before using it as a solubility-aiding

agent for chelidonine. Thirty planarians were exposed to DMSO in water at different concentrations (0.025%, 0.05%, 0.1%) or maintained in water (control group). The animals were exposed to these experimental conditions for a period of 7 days (long-term DMSO exposure). To assess the effects of DMSO during regeneration, thirty planarians were exposed to the same treatment, but were transected at the prepharyngeal region (Fig. 1) after 1 day of exposure and left to regenerate in different concentrations of DMSO or in water. Chelidonine (Sigma) was freshly prepared in DMSO for each experiment, so that the final concentration of DMSO in water was 0.025%, 0.05% and 0.1% (corresponding to 5μM chelidonine, 10μM chelidonine and 20μM chelidonine, respectively). Thirty intact worms of similar body size were exposed by soaking to the drug for 3-4 days. In another experimental protocol the animals were transected at the prepharyngeal region after 1 day of treatment and left to regenerate in presence of chelidonine or DMSO (control group). The fragments were sacrificed 3 or 6 days after cutting for successive experiments. In each protocol the solutions (30 ml) were changed daily.

2.2 Preparation of dissociated cells

Dissociated planarian cells were prepared according to Baguñà and Romero (1981) and stained with Hoechst 33342 DNA dye. A total of 10 μl of cell suspension was used to calculate the number of metaphase cells *versus* anaphase/telophase cells. The number of mitotic figures was normalized to the number of total cells and the values are average ± s.d. of six independent samples, analyzed in duplicate for each experiment, assuming as 100% the value of control planarians.

2.3. RNA isolation, reverse transcription and real time RT-PCR

Total RNA was isolated using the Nucleospin RNAII kit (Macherey-Nagel), according to the manufacturer's instructions. Before cDNA synthesis, each extraction was tested for the absence of genomic DNA. cDNA was synthesized using GoTaq® 2-step RT-qPCR system (Promega). For each experiment nine different RNA extractions were performed with three independent experimental groups. Each RNA sample was obtained from an experimental group including 6 different regenerating fragments (3 head fragments + 3 tail fragments). Real time RT-PCR was performed at least three times for each examined gene, using three replicates for each cDNA. SYBR Green chemistry-based RT-PCR was carried out on a Rotor-Gene 6000 Real time-PCR (Corbett Research). Details of the procedures are given in Table S1, according to the MIQE guidelines (Bustin et al., 2009). Preliminary experiments were performed to evaluate the transcriptional stability of candidate endogenous reference genes (Yuwen et al., 2010). Under our experimental conditions (0.025%, 0.05%, 0.1% DMSO), the stability of two planarian endogenous reference genes, *D. japonica elongation factor-2* (*DjEF2*) and *D. japonica β-actin* (*DjACTB*) was analyzed using NormFinder software. *DjEF2* was the most stable gene and for this reason it was selected as reference for comparative gene expression analysis. Expression levels of *DjEF2* and *DjACTB* reference genes after DMSO treatments were analyzed by real time RT-PCR (Fig. S1).

2.4. Statistics

All values are presented as mean ± standard deviation (s.d.) of at least three different experiments in triplicate. Data tabulation and descriptive statistics were performed with Microsoft Excel. The statistical analysis was performed by means of non-parametric ANOVA Kruskal-Wallis test and Student's t-test (data not shown). According to Shapiro-

Wilk test, the distribution of the logarithmically transformed data was not significantly different from that of a normal distribution. In addition, homoscedasticity analysis, determined with Hartley's F max test, revealed no differences in variability between the groups (in all samples the F max calculated was lower than the critical value at 0.05). For mitosis analysis, statistical significance was determined using the two-tailed unpaired Student's t-test. A value of $P<0.05$ was considered statistically significant.

2.5. In situ hybridization

Whole mount *in situ* hybridization was performed according to Umesono et al. (1997) with minor modifications (Nogi and Levin, 2005). DIG-labeled antisense riboprobe was synthesized by RNA polymerase (Roche), employing linearized *Djmcm2* (cDNA: region 168 to 761 bp) in pGEM-T Easy vector (Promega) as the template.

3. Results

3.1. Effects of chelidonine on tissue homeostasis and regeneration

Under our experimental conditions, 20μM chelidonine treatment resulted in abnormal phenotypes. On the contrary, no effects on mortality or gross abnormalities were observed in DMSO-treated planarians that appeared similar to the control group maintained in water. Most of the 20μM chelidonine-treated planarians exhibited lesions and ventral curling (Fig. 2A-D). This phenotype, although consistent with a stress effect, strongly resembles the stereotypical stem cell-defective phenotype observed when stem cells are selectively eliminated by γ-irradiation or when animals are subjected to RNAi for genes required for stem cell function (Guo et al., 2006; Reddien et al., 2005). Chelidonine-induced phenotypes began to be detected around day 3. Some of them died

after the transfer in water, while others (about 80%) recovered a normal morphology in a few days. As these data suggest that 20μM chelidonine interferes with proper stem cell function during tissue homeostasis, we also analyzed the effects during regeneration. When animals were transected after 1 day from the beginning of the treatment, 0.1% DMSO controls completely regenerated the missing body parts (Fig. 2E,F; Fig. S2A,B) while regeneration was severely impaired, or minimal blastemal tissue, often with blisters, was produced in chelidonine-treated fragments (Fig. 2G,H; Video S1 and Video S2). This phenotype was not dependent on which body part was regenerating, because a head that was regenerating a new tail, or a tail that was regenerating a new head, or a trunk regenerating both a head and a tail, showed similar defects (data not shown). Similar to that observed in intact animals, most chelidonine-treated fragments rescued regenerative capability after transfer in water. Further experiments with different chelidonine concentrations were performed. The 10μM and 5μM chelidonine treatment did not produce visible morphological alterations (data not shown). On the whole, these results indicate that the action of chelidonine is dose-dependent within the concentration range examined.

3.2. Molecular analysis of planarian cells by assessment of potential changes in gene expression level

Molecular analysis of stem cells and their descendants, as well as of specific cell types can be performed in planarians by gene expression analysis of different markers. We therefore examined the effects of chelidonine using *Djmcm2,* a planarian gene encoding an essential DNA replication factor, as a molecular marker for proliferating cells (Salvetti et al., 2000). This protein is a component of a complex - abundant in proliferating cells - that forms at the origins of replication during the initiation step of DNA replication and is

essential for ensuring that the process takes place only once for each cell cycle. Although whole mount *in situ* hybridization is not a quantitative method, our results showed a reduction of *Djmcm2* hybridization signal in 20μM chelidonine-treated planarians when compared to controls soaked in 0.1% DMSO (Fig. 3A-C). No morphological differences were detected between planarians soaked in DMSO (0.025%, 0.05%, 0.1%) and controls maintained in water. Both planarian fragments regenerated a blastema of similar size and showed comparable *Djmcm2* expression pattern (Fig. S2A,B). To evaluate possible effects of long-term exposure to different DMSO concentrations on cell proliferation, *Djmcm2* expression level was also investigated by real time RT-PCR in regenerating planarians. The level of *Djmcm2* transcripts after DMSO treatments was similar to that of the controls in water, indicating that this solvent does not significantly influence *in vivo* cell proliferation (Fig. S2C). The level of *Djmcm2* expression after 20μM chelidonine treatment was compared to 0.1% DMSO controls. A significant decrease of *Djmcm2* expression level was observed. This result clearly demonstrates an anti-proliferative effect of chelidonine on planarian stem cells. Real time RT-PCR data showed that 5μM chelidonine and 10μM chelidonine did not cause any significant reduction of *Djmcm2* expression level (Fig. 4A-C). An ortholog of *nanos*, a gene required for maintenance of germ cell identity in diverse organisms, was also used as a specific marker to study the effect of 20μM chelidonine on the germline stem cell subpopulation. Although primordial germ cells represent precursors of eggs and sperms in all sexually reproducing species, also asexual planarians express *nanos* and produce primordial germ cells, even if yet unknown mechanisms prevent these cells from developing into functional gametes (Handberg-Thorsager and Saló, 2007; Wang et al., 2007; Sato et al., 2006). Our results demonstrate that 20μM chelidonine causes a significant

downregulation of the planarian *nanos* (*Djnos*) expression level (Fig. S3A). This result implies that the overall stem cell system (the sole cells with proliferative capability in planarians) is a selective target of the drug. To support this possibility, the change in expression level of *Djinx-11*, a marker of committed progenitors, has been analyzed by real time RT-PCR. *Djinx-11* gene encodes a planarian stem cell gap junction protein required for the movement of small molecules that control maintenance, migration and differentiation of the progeny of proliferative cells (Oviedo and Levin, 2007). The expression level of this gene did not significantly change after chelidonine treatments (Fig. 4D-F). To evaluate possible cytotoxic side effects of this alkaloid to postmitotic cells, another member of the innexin gene family, *Djinx1*, whose expression is limited to the intestine, was examined (Oviedo and Levin, 2007). *Djsyt*, a planarian gene encoding synaptotagmin, a protein involved in the regulation of synaptic and extra-synaptic membrane trafficking in the central nervous system, was also used as a marker specific for nerve cells. Our data show that chelidonine treatments do not affect the expression level of these genes (Fig. S3B; Fig. 4G-I). On the whole, these results imply that chelidonine exposure causes no effects on differentiated cell types, such as intestinal or nerve cells.

3.3 Chelidonine, at a concentration of 20μM, prevents cell cycle progression of stem cells

To visualize in detail the effects of chelidonine on cell cycle and morphology of planarian cells, macerates (i.e. single cell types obtained by cell dissociation of worms: Baguñà and Romero, 1981) have been examined in specimens exposed to 20μM chelidonine and compared to macerates of control planarians treated with 0.1% DMSO. Macerates from planarian head and tail fragments (3 days of regeneration) were used in order to maximize the number of mitotic figures in the samples. Chelidonine treatment affected

proliferating cells, resulting in abnormal metaphase and anaphase/telophase figures, as visualized by counterstaining with Hoechst 33342 in Fig. 5A-F. Treated interphase cells were unaffected (data not shown). To assess the possibility that cells exposed to chelidonine do not progress in the cell cycle, the percentage of metaphase and anaphase/telophase figures has been compared in macerates obtained from both chelidonine-treated and DMSO control planarians. As shown in Fig. 5G the percentage of cycling cells, able to proceeds to anaphase/telophase, appeared significantly reduced in planarians treated with chelidonine with respect to the controls. Under the anti-proliferative effect of chelidonine, in fact, the percentage of metaphase figures, calculated by the analysis of the ratio between metaphases and anaphases/telophases in 0.1% DMSO controls and 20μM chelidonine, resulted significantly higher in the treated specimens that in the controls. The simplest explanation of these results is that a number of cycling cells remain arrested at metaphase.

4. Discussion

In this work, we demonstrate the anti-proliferative effect of chelidonine for the first time on stem cells *in vivo*, using planarians as an animal model. Chelidonine is one of the principal alkaloids obtained from *C. majus*, a plant with a broad spectrum of pharmacological activities. Although chelidonine lacks DNA-binding capacity, this alkaloid possesses significant anti-proliferative effects, and has the ability to arrest cell growth in different cell lines. These results support the possibility that chelidonine is an effective anticancer drug (Kaminskyy et al., 2008; Philchenkov et al., 2008). Different mechanisms have been proposed to explain the properties of this drug. Inhibition of telomerase activity and apoptosis stimulation has been described in primary human uveal

melanoma cells **(**Kemény-Beke et al., 2006; Noureini and Wink, 2009). Literature data also demonstrate that chelidonine blocks mitosis by inhibition of tubulin polymerization and activation of the stress activated protein kinase/Jun kinase pathway (Panzer et al., 2001; Wolff and Knipling, 1993). Although these effects have been proved in different cell lines, no data are available on stem cells *in vivo***.** Planarians are highly plastic animals (Gentile et al., 2011; Wagner et al., 2011). During regeneration planarian stem cells activate an intense proliferative program to restore the lost body parts. The presence of two distinct cellular compartments (a proliferative compartment and a functional, postmitotic compartment), as well as the use of cell-specific markers, makes regeneration a unique experimental model to predict drug-induced effects on single cell types. We have demonstrated that 20μM chelidonine treatment causes morphological defects in these animals, and also prevents regeneration, mimicking the phenotypic effects produced by irradiation or functional ablation of stem cell genes (Guo et al., 2006; Reddien et al., 2005). These results raise the possibility that chelidonine affects viability of stem cells. However, we observed that the chelidonine-induced effects were reversible, as most of the treated specimens recovered a normal morphology and regenerative capability after the transfer in water. This finding suggests that partial reduction in the number of stem cells occurred. Survived stem cells could then repopulate the body of the animals in a short time (Salvetti et al., 2009, Wagner et al., 2011). Our results support the possibility that this alkaloid blocks cell proliferation with limited cytotoxic effects (Kaminskyy et al., 2008). Although precise calibration of a drug concentration *in vivo* is difficult to achieve, especially when administered by soaking, we observed that the anti-proliferative effects of chelidonine depended on the applied dose. Anti-proliferative drugs are usually administered *in vivo* at a concentration required to completely halt the growth of target cells (total growth inhibition: TGI). The most effective treatment on planarians was

20μM chelidonine. This value is comparable to chelidonine TGI on several tumor cell lines. A wide screening on 60 different tumor cell lines performed at NIH shows, in fact, a TGI for chelidonine of 20μM (Developmental Therapeutics Program NCI/NIH, website http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp, searching for "chelidonine" and selecting NCI60 Cancer Screen Current Data).

In order to develop chelidonine to its full potential clinical applications, it is pivotal to obtain a clearer understanding of the molecular mechanisms that underlie its effects on stem cells *in vivo*. Because these studies are difficult in mammalian models, planarians and their pluripotent stem cells represent a suitable complementary system for dissecting relevant molecular details. We have followed a molecular strategy to analyze and link variation of gene expression with drug response of specific cell types. The use of molecular markers that characterize proliferating cells demonstrates that 20μM chelidonine provokes a significant diminution in the expression level of these genes. Lack of side effects produced by DMSO long-term exposure has been proved by whole mount *in situ* hybridization and real time RT-PCR on regenerating planarians exposed to different DMSO concentrations (0.025%-0.1%) or maintained in water. On the whole the results demonstrate that DMSO i) does not cause morphological defects, ii) does not interfere with regenerative capability or with *Djmcm2* expression and prove that the observed dose-effect is related to an increase in chelidonine concentrations and not in DMSO concentrations, suggesting that the effects of DMSO and chelidonine are not cumulative. It is important to point out that the effect of the drug extends to different stem cell subpopulations, as demonstrated by the significant decrease of the *Djnos*-positive germline stem cells in chelidonine-treated animals. These results provide evidence that 20μM dose reduces significantly cell growth and affects germline stem cell viability. No effects were detected using markers generally employed to characterize differentiated cell

types, such as nerve or intestinal cells, suggesting that no generalized cytotoxic effect occurred. These results represent the first gene expression analysis in relation to chelidonine activity *in vivo* and reveal that the drug specifically affects dividing stem cells, whereas postmitotic cells are not influenced.

We sought to understand how chelidonine interferes with the planarian stem cell system. Our data demonstrate that the effects of chelidonine on cell cycle progression may depend on inhibition of tubulin polymerization. A significant arrest of cells in the mitotic metaphase was observed as a consequence of chelidonine treatment. The inhibitory effect on dividing cells resulted in fact in abnormal accumulation of metaphase figures, while the incidence of anaphases/telophases consistently decreased. In addition, numerous anomalous mitotic cell forms could be observed. A chelidonine-mediated partial inactivation of the mitotic spindle, with consequent cytostatic effects on root tip cells (i.e. cells with stem-cell-like properties), has been described in *Allium cepa* (Krahulcová, 1979). It is possible that this alkaloid acts at the colchicine-binding site, as it is a weak competitive inhibitor of colchicine binding (Wolff and Knipling, 1993). Even though these studies varied in methodology and sample, the results are remarkably consistent with our results, suggesting that the inhibitory effect of chelidonine on tubulin polymerization represents a major mechanism for growth arrest of dividing cells *in vivo*. The results of this research, according with previous studies on cell lines, support the idea that chelidonine represents an important anti-proliferative alkaloid devoid of significant cytotoxicity and emphasize the potential value of planarian stem cells in finding better drugs to treat human disease.

5. Conclusions

To the best of our knowledge, this study is the first to report the effects of chelidonine on arrest of cell cycle in stem cells *in vivo*. Planarian model is also a rich source of information about the mechanisms of cell growth inhibition. Based on our data we hypothesize a mechanistic scenario that predicts how inhibition of tubulin polymerization plays a key role in the function of this drug. These findings provide new information to complete the understanding of the chelidonine anti-proliferative effects, and support the possible potential of this drug for the therapy of hyper-proliferative disorders, such as cancer and psoriasis.

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Figure legends

Figure 1. Schematic of amputation procedure of a planarian. Black line indicates the amputation level. Complete regeneration of the missing body parts occurs in about 10 days at 18°C. Regenerated body parts are shown in light grey. ph: pharynx.

Figure 2. Brightfield images of planarians after 20μM chelidonine treatment. (A) Schematic representation of an intact planarian. (B) An intact control planarian 0.1% DMSO. (C) An intact planarian after 3 days of chelidonine treatment shows body lesions. (D) An intact planarian after 3 days of chelidonine treatment shows ventral curling. (E) Schematic representation of a planarian tail fragment regenerating a head. (F) A 0.1% DMSO control tail fragment regenerating a head, 3 days after amputation. (G) A tail fragment treated with chelidonine did not form regeneration blastema, 3 days after amputation. (H) A tail fragment treated with chelidonine shows a very reduced blastema with blisters, 3 days after amputation. Anterior is up. Dashed line in D and E corresponds to the original amputation site. Scale bar: 1 mm.

Figure 3. *In situ* hybridization with *Djmcm2* of planarian head fragments after 20μM chelidonine treatment. (A) Schematic representation of a planarian head fragment regenerating a tail. (B) A 0.1% DMSO control regenerating fragment shows strong hybridization signal, visualized as brown color by Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) chromogen precipitation in the mesenchymal tissue, where planarian stem cells are located. (C) A comparable fragment hybridized after chelidonine treatment shows reduced *Djmcm2* expression. Six days of regeneration. Anterior is up. Scale bar: 0.5 mm.

Figure 4. Expression level of *Djmcm2*, *Djinx-11* and *Djsyt* analyzed in planarians after chelidonine treatment by real time RT- PCR. (A-C) *Djmcm2* expression level**.** **(**A) 0.025% DMSO controls (DMSO) and 5μM chelidonine (CH). (B) 0.05% DMSO controls (DMSO) and 10μM chelidonine (CH). (C) 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). (D-F) *Djinx-11* expression level. **(**D) 0.025% DMSO controls (DMSO) and 5μM chelidonine (CH). (E) 0.05% DMSO controls (DMSO) and 10μM chelidonine (CH). (F) 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). (G-I) *Djsyt* expression level. **(**G) 0.025% DMSO controls (DMSO) and 5μM chelidonine (CH). (H) 0.05% DMSO controls (DMSO) and 10μM chelidonine (CH). (I) 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). Expression levels are indicated in relative units, assuming a value of 1 for DMSO-treated controls. Samples were compared using the Kruskal-Wallis non-parametric ANOVA. **P<0.01.

Figure 5: Mitotic abnormalities arise *in vivo* in response to 20μM chelidonine treatment.

(A-C) Mitotic figures after 0.1% DMSO treatment (DMSO). (D-F) Mitotic figures after 20μM chelidonine treatment (CH). (A**)** Metaphase chromosomes appear arranged in the middle of the cell on the metaphase plate before being separated into each of the two daughter cells at the anaphase stage. (B) A cell at anaphase, usually defined as the separation of the sister chromatids by shortening of the microtubules of the mitotic spindle. (C) A telophase figure: both sets of chromosomes become now surrounded by new nuclei and unfold back into chromatin, while cytokinesis completes cell division. (D) A metaphase figure after chelidonine treatment. The chromosomes appear abnormally arranged in the cell. (E) An anaphase figure after chelidonine treatment, showing altered

chromosome separation. (F) A telophase figure after chelidonine treatment. In this cell cytokinesis did not occur to separate the daughter cells, resulting in a cell containing two condensed groups of chromosomes. Scale bar: 10 μm. (G) Analysis of the ratio between metaphases and anaphases/telophases in 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). Values are expressed as percentages with respect to controls, to which the arbitrary value of 100% has been assigned. Each value represents the mean ± standard deviation of six independent samples counted in duplicate. Samples were compared using t- test. **P<0.001.

Supporting information

Figure S1: **Expression levels of *DjEF2* and *DjACTB* reference genes after DMSO treatment, analyzed by real time RT-PCR.** (A) *DjACTB*. (B) *DjEF2*. Expression levels are indicated in relative units, assuming a value of 1 for water-treated controls.

Figure S2. Effect of different DMSO concentrations, compared to water as a negative control. (A,B) *Djmcm2* expression as visualized by situ hybridization of tail fragments regenerating a head, 6 days of regeneration. (A) A fragment maintained in water. (B) A fragment in 0.1% DMSO. Comparison of the obtained results demonstrates that both fragments regenerate normal heads and show similar *Djmcm2* expression. Dashed line in A and B corresponds to the original amputation site. (C) Expression level of *Djmcm2* compared by real time RT-PCR in regenerating planarians (3 days of regeneration), exposed to different DMSO concentrations (0.025%-0.1%) or maintained in water. Expression levels are indicated in relative units, assuming a value of 1 for water-treated controls.

Figure S3: **Expression level of *Djnos* and *Djinx1* analyzed in planarians after 20μM chelidonine treatment by real time RT-PCR.** (A) *Djnos* expression level in chelidonine-treated planarians (CH) and 0.1%DMSO controls. (B) *Djinx1* expression level in chelidonine-treated planarians (CH) and DMSO 0.1%controls. Expression levels are indicated in relative units, assuming a value of 1 for DMSO-treated controls. Samples were compared using the Kruskal-Wallis non-parametric ANOVA. *$P<0.05$.

Video S1: A representative example of a ‘curled’ planarian fragment, as produced by 20μM chelidonine treatment. A small blastema, with a blister, is barely visible at the anterior region, 3 days after amputation (600 frames, 66.7 ms per frame).

Video S2: A representative example of a 0.1% DMSO regenerating control planarian is shown for comparison. Blastema is visible as a white area at the anterior region, 3 days after amputation (510 frames, 66.7 ms per frame).

Figure 1
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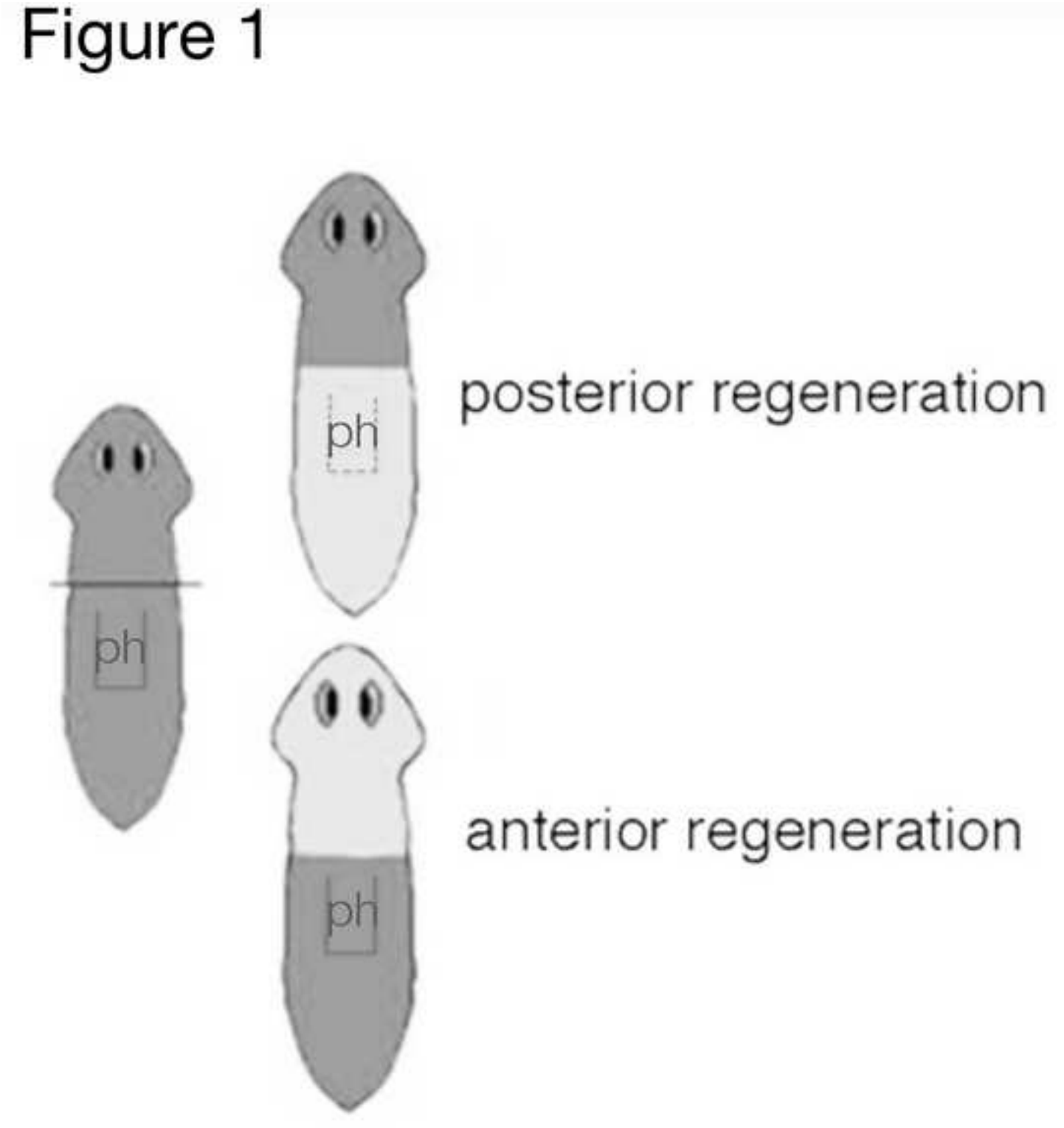

Figure 2

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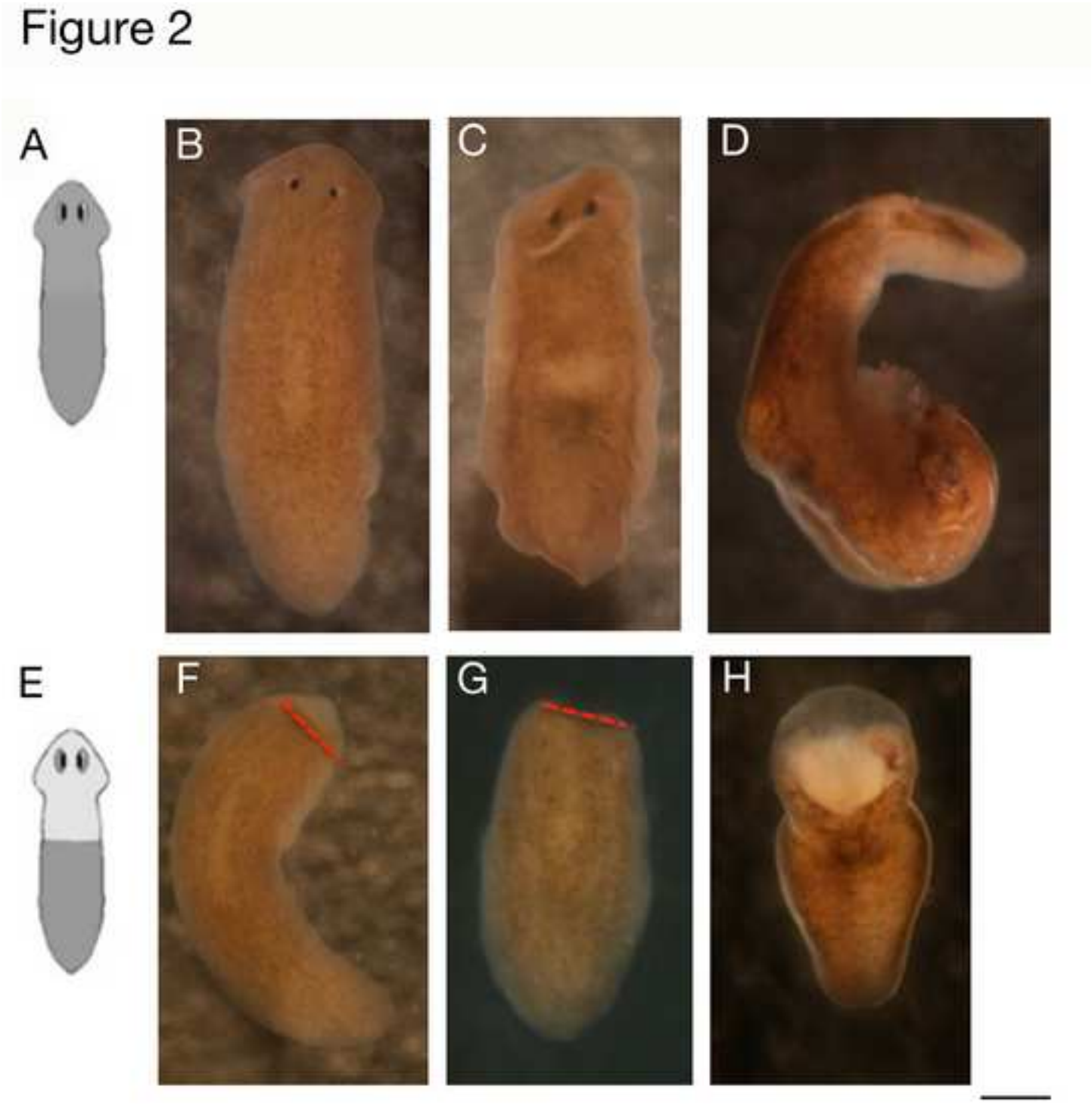

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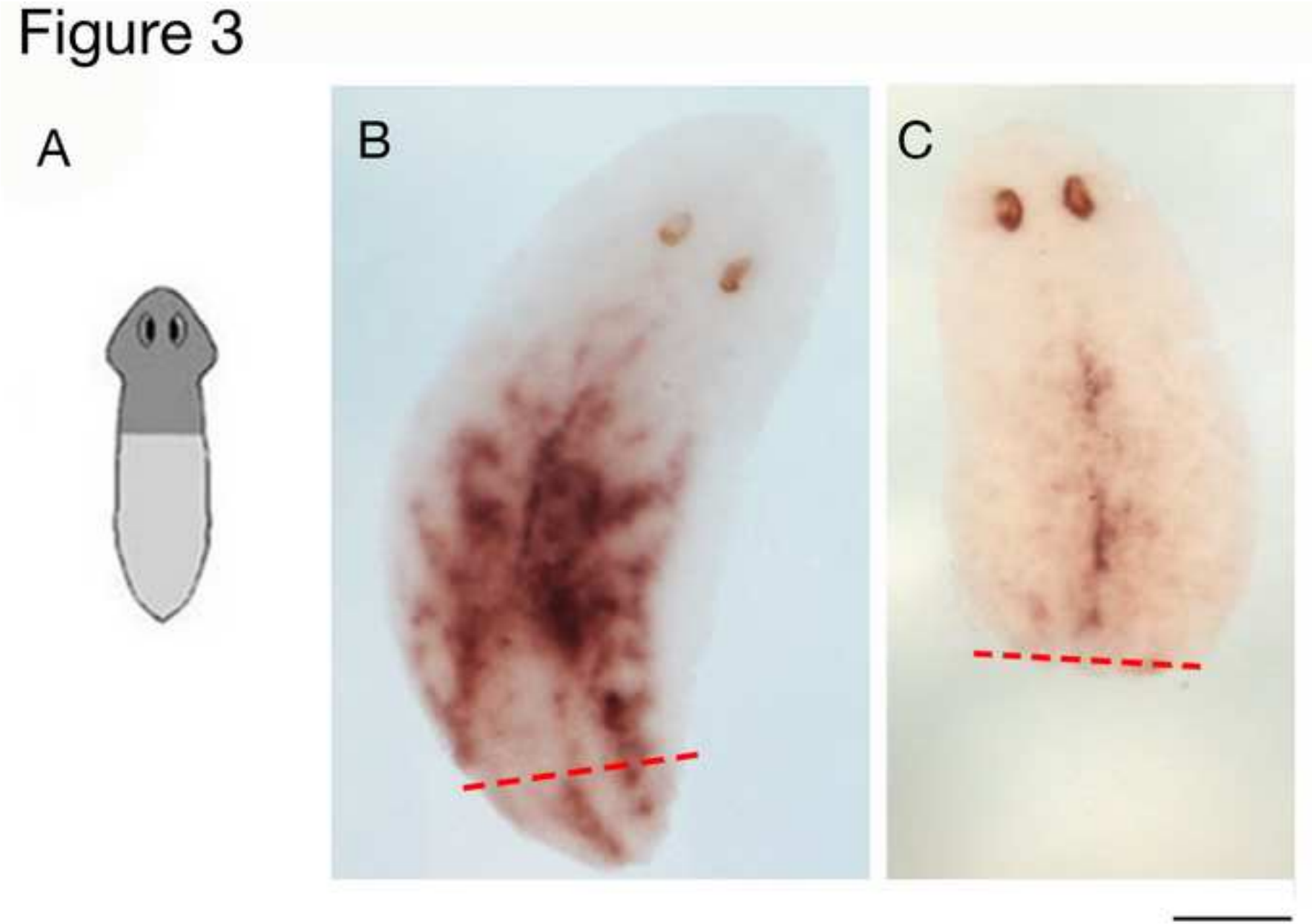

Figure 4

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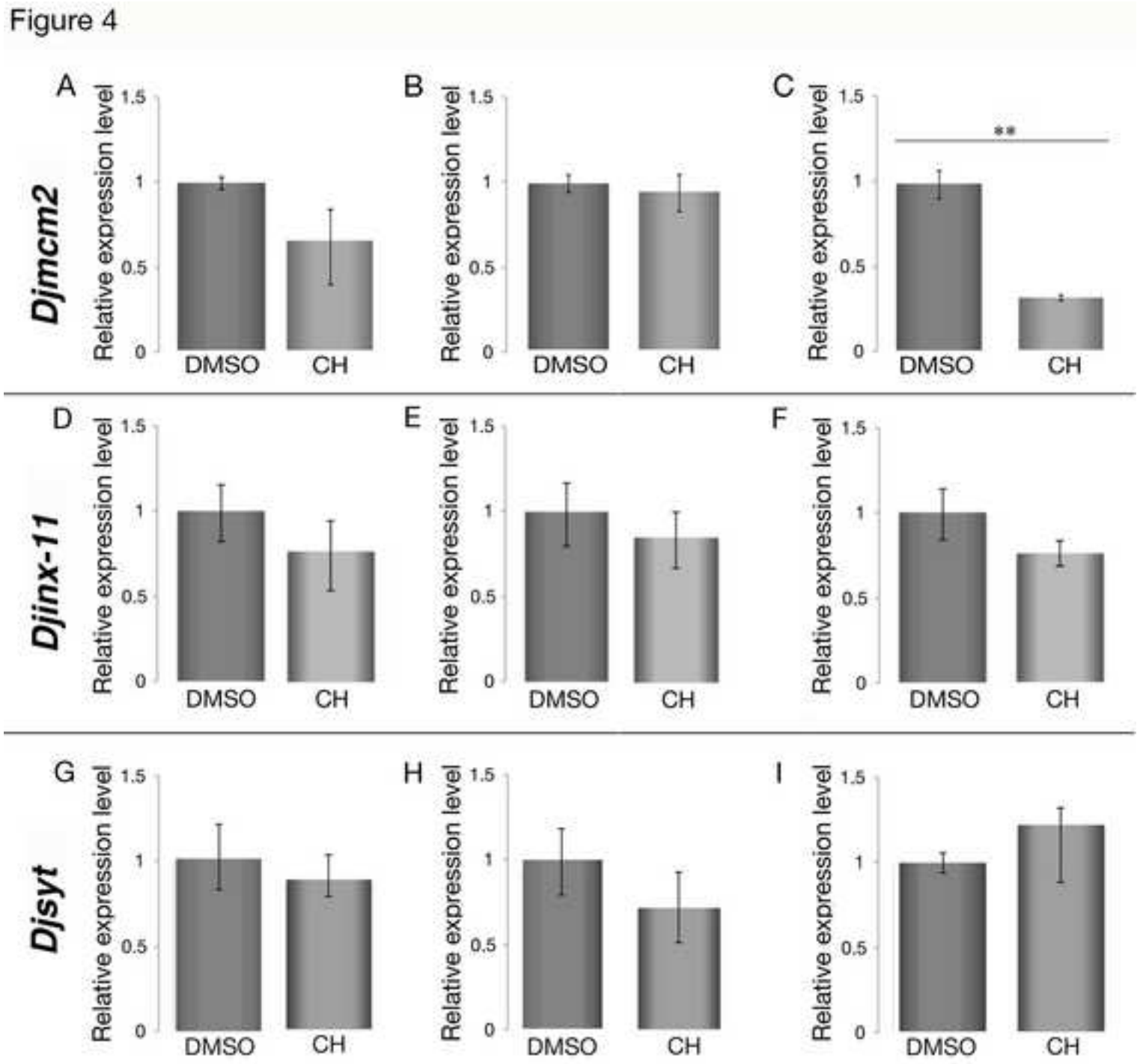

Figure 5

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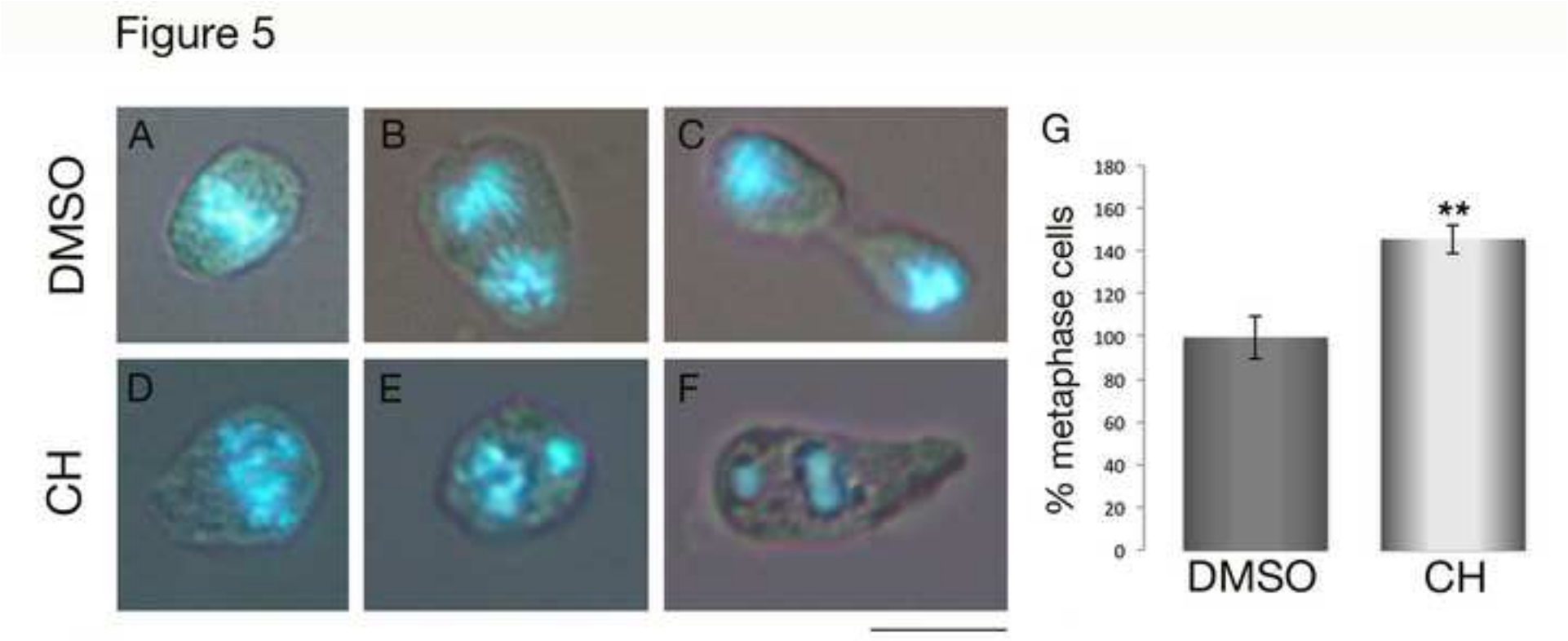

The *in vivo* effect of chelidonine on the stem cell system of planarians

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Abstract

The presence of adult pluripotent stem cells and the amazing regenerative capabilities make planarian flatworms an extraordinary experimental model to assess *in vivo* the effects of substances of both natural and synthetic origin on stem cell dynamics. This study focuses on the effects of chelidonine, an alkaloid obtained from *Chelidonium majus*. The expression levels of molecular markers specific for stem or differentiated cells were compared in chelidonine-treated and control planarians. The use of these markers demonstrates that chelidonine produces *in vivo* a significant anti-proliferative effect on planarian stem cells in a dose-dependent fashion. In response to chelidonine treatment mitotic abnormalities were also observed and the number of cells able to proceed to anaphase/telophase appeared significantly reduced with respect to the controls. Our results support the possibility that chelidonine acts on cell cycle progression by inhibition of tubulin polymerization. These studies provide a basis for preclinical evaluation *in vivo* of the effects of chelidonine on physiologically proliferating stem cells.

Keywords: Chelidonine, Planarian, Stem Cells, Cell proliferation, Molecular Markers

1. Introduction

Stem cells are, by definition, undifferentiated cells that have both the ability to self-renew, making identical copies at each division, and to differentiate specialized cells. These cells offer the possibility of a renewable source of replacement cells and tissues, with potential applications in regenerative medicine. However, a number of pathological conditions, such as cancer and psoriasis, are characterized by hyper-proliferative phenomena. Consequently, inhibition of cell proliferation results to be potentially useful in drug discovery to design agents targeted to the treatment of diseases characterized by uncontrolled or abnormal cellular proliferation. In this context, the so-called adult or somatic stem cells (ASCs) have attracted special attention. *In vivo* studies of ASCs are not easy in mammals due to the difficulty of developmental biology studies *in vivo* using vertebrate models. For this reason, most of the knowledge on ASC biology results from *in vitro* studies. Non-mammalian model systems can be alternatively used to assess *in vivo* the effect of substances on these cells.

Planarians are an ideal animal model for *in vivo* analysis of ASCs. Their extraordinary ability to regenerate, as well as to sustain continuous homeostatic turnover of all differentiated cell types, relies on the presence of stem cells that share characteristics with the stem cells of higher organisms, including humans. Planarian regeneration involves remodeling of pre-existing tissues and formation of an outgrowth at wound region, in which the missing parts are newly formed (the regenerative blastema) (Gentile et al., 2011; Slack, 2011). The discovery that planarian stem cells use evolutionarily conserved mechanisms that are surprisingly similar to the mechanisms we observe in mammalian stem cells, makes these invertebrates suitable to screen the effects of new drugs on stem

cells. In addition, the planarian genome shares a large number of genes with the human genome, including sequences involved in various aspects of human biology and disease. Our work focuses on the analysis of the effects produced *in vivo* by substances of natural origin, particularly a series of compounds contained in *Chelidonium majus*, on planarian stem cells. *C. majus* (greater celandine) is a herbaceous plant of the *Papaveraceae* family that spontaneously grows in temperate areas. As a starting point we studied the effects of chelidonine, an isoquinoline alkaloid derived from *C. majus*. The pharmacological potential of mitotic arrest by chelidonine is considered promising in cancer therapy. In this paper the activity of chelidonine was tested *in vivo* in *Dugesia japonica*, a clonal planarian strain widely used to study drug responsiveness (Kitamura et al., 2003; Nogi and Levin, 2005; Nogi et al., 2009).

2. Materials and methods

2.1. Animals and treatments

Asexual specimens of *D. japonica* (GI strain) were maintained at 18°C in autoclaved stream water, fed weekly with chicken liver and starved one week before the experiments. Control groups were represented by planarians soaked in dimethylsulfoxide (DMSO) at different concentrations in the absence of drug and by untreated planarians, maintained in water. As literature data demonstrate that DMSO concentrations >0.1% should be avoided in order to be able to reliably observe any behavioral or toxic effects of hydrophobic drugs in these animals (Pagán et al., 2006; Yuan et al., 2011), we first analyzed the long-term effects of DMSO exposure before using it as a solubility-aiding

agent for chelidonine. Thirty planarians were exposed to DMSO in water at different concentrations (0.025%, 0.05%, 0.1%) or maintained in water (control group). The animals were exposed to these experimental conditions for a period of 7 days (long-term DMSO exposure). To assess the effects of DMSO during regeneration, thirty planarians were exposed to the same treatment, but were transected at the prepharyngeal region (Fig. 1) after 1 day of exposure and left to regenerate in different concentrations of DMSO or in water. Chelidonine (Sigma) was freshly prepared in DMSO for each experiment, so that the final concentration of DMSO in water was 0.025%, 0.05% and 0.1% (corresponding to 5μM chelidonine, 10μM chelidonine and 20μM chelidonine, respectively). Thirty intact worms of similar body size were exposed by soaking to the drug for 3-4 days. In another experimental protocol the animals were transected at the prepharyngeal region after 1 day of treatment and left to regenerate in presence of chelidonine or DMSO (control group). The fragments were sacrificed 3 or 6 days after cutting for successive experiments. In each protocol the solutions (30 ml) were changed daily.

2.2 Preparation of dissociated cells

Dissociated planarian cells were prepared according to Baguñà and Romero (1981) and stained with Hoechst 33342 DNA dye. A total of 10 μl of cell suspension was used to calculate the number of metaphase cells *versus* anaphase/telophase cells. The number of mitotic figures was normalized to the number of total cells and the values are average ± s.d. of six independent samples, analyzed in duplicate for each experiment, assuming as 100% the value of control planarians.

2.3. RNA isolation, reverse transcription and real time RT-PCR

Total RNA was isolated using the Nucleospin RNAII kit (Macherey-Nagel), according to the manufacturer's instructions. Before cDNA synthesis, each extraction was tested for the absence of genomic DNA. cDNA was synthesized using GoTaq® 2-step RT-qPCR system (Promega). For each experiment nine different RNA extractions were performed with three independent experimental groups. Each RNA sample was obtained from an experimental group including 6 different regenerating fragments (3 head fragments + 3 tail fragments). Real time RT-PCR was performed at least three times for each examined gene, using three replicates for each cDNA. SYBR Green chemistry-based RT-PCR was carried out on a Rotor-Gene 6000 Real time-PCR (Corbett Research). Details of the procedures are given in Table S1, according to the MIQE guidelines (Bustin et al., 2009). Preliminary experiments were performed to evaluate the transcriptional stability of candidate endogenous reference genes (Yuwen et al., 2010). Under our experimental conditions (0.025%, 0.05%, 0.1% DMSO), the stability of two planarian endogenous reference genes, *D. japonica elongation factor-2* (*DjEF2*) and *D. japonica β-actin* (*DjACTB*) was analyzed using NormFinder software. *EF2* was the most stable gene and for this reason it was selected as reference for comparative gene expression analysis. Expression levels of *DjEF2* and *DjACTB* reference genes after DMSO treatments were analyzed by real time RT-PCR (Fig. S1).

2.4. Statistics

All values are presented as mean ± standard deviation (s.d.) of at least three different experiments in triplicate. Data tabulation and descriptive statistics were performed with Microsoft Excel. The statistical analysis was performed by means of non-parametric ANOVA Kruskal-Wallis test. For mitosis analysis, statistical significance was determined

using the two-tailed unpaired Student's t-test. A value of $P<0.05$ was considered statistically significant.

2.5. In situ hybridization

Whole mount *in situ* hybridization was performed according to Umesono et al. (1997) with minor modifications (Nogi and Levin, 2005). DIG-labeled antisense riboprobe was synthesized by RNA polymerase (Roche), employing linearized *Djmcm2* (cDNA: region 168 to 761 bp) in pGEM-T Easy vector (Promega) as the template.

3. Results

3.1. Effects of chelidonine on tissue homeostasis and regeneration

Under our experimental conditions, 20μM chelidonine treatment resulted in abnormal phenotypes. On the contrary, no effects on mortality or gross abnormalities were observed in DMSO-treated planarians that appeared similar to the control group maintained in water. Most of the 20μM chelidonine-treated planarians exhibited lesions and ventral curling (Fig. 2A-D). This phenotype, although consistent with a stress effect, strongly resembles the stereotypical stem cell-defective phenotype observed when stem cells are selectively eliminated by γ-irradiation or when animals are subjected to RNAi for genes required for stem cell function (Guo et al., 2006; Reddien et al., 2005). Chelidonine-induced phenotypes began to be detected around day 3. Some of them died after the transfer in water, while others (about 80%) recovered a normal morphology in a few days. As these data suggest that 20μM chelidonine interferes with proper stem cell function during tissue homeostasis, we also analyzed the effects during regeneration. When animals were transected after 1 day from the beginning of the treatment, 0.1%

DMSO controls completely regenerated the missing body parts (Fig. 2E,F; Fig. S2A,B) while regeneration was severely impaired, or minimal blastemal tissue, often with blisters, was produced in chelidonine-treated fragments (Fig. 2G,H; Video S1 and Video S2). This phenotype was not dependent on which body part was regenerating, because a head that was regenerating a new tail, or a tail that was regenerating a new head, or a trunk regenerating both a head and a tail, showed similar defects (data not shown). Similar to that observed in intact animals, most chelidonine-treated fragments rescued regenerative capability after transfer in water. Further experiments with different chelidonine concentrations were performed. The 10μM and 5μM chelidonine treatment did not produce visible morphological alterations (data not shown). On the whole, these results indicate that the action of chelidonine is dose-dependent within the concentration range examined.

3.2. Molecular analysis of planarian cells by assessment of potential changes in gene expression level

Molecular analysis of stem cells and their descendants, as well as of specific cell types can be performed in planarians by gene expression analysis of different markers. We therefore examined the effects of chelidonine using *Djmcm2,* a planarian gene encoding an essential DNA replication factor, as a molecular marker for proliferating cells (Salvetti et al., 2000). This protein is a component of a complex - abundant in proliferating cells - that forms at the origins of replication during the initiation step of DNA replication and is essential for ensuring that the process takes place only once for each cell cycle. Although whole mount *in situ* hybridization is not a quantitative method, our results showed a reduction of *Djmcm2* hybridization signal in 20μM chelidonine-treated

planarians when compared to controls soaked in 0.1% DMSO (Fig. 3A-C). No morphological differences were detected between planarians soaked in DMSO (0.025%, 0.05%, 0.1%) and controls maintained in water. Both planarian fragments regenerated a blastema of similar size and showed comparable *Djmcm2* expression pattern (Fig. S2A,B). To evaluate possible effects of long-term exposure to different DMSO concentrations on cell proliferation, *Djmcm2* expression level was also investigated by real time RT-PCR in regenerating planarians. The level of *Djmcm2* transcripts after DMSO treatments was similar to that of the controls in water, indicating that this solvent does not significantly influence *in vivo* cell proliferation (Fig. S2C). The level of *Djmcm2* expression after 20μM chelidonine treatment was compared to 0.1% DMSO controls. A significant decrease of *Djmcm2* expression level was observed. This result clearly demonstrates an anti-proliferative effect of chelidonine on planarian stem cells. Real time RT-PCR data showed that 5μM chelidonine and 10μM chelidonine did not cause any significant reduction of *Djmcm2* expression level (Fig. 4A-C). An ortholog of *nanos*, a gene required for maintenance of germ cell identity in diverse organisms, was also used as a specific marker to study the effect of 20μM chelidonine on the germline stem cell subpopulation. Although primordial germ cells represent precursors of eggs and sperms in all sexually reproducing species, also asexual planarians express *nanos* and produce primordial germ cells, even if yet unknown mechanisms prevent these cells from developing into functional gametes (Handberg-Thorsager and Saló, 2007; Wang et al., 2007; Sato et al., 2006). Our results demonstrate that 20μM chelidonine causes a significant downregulation of the planarian *nanos* (*Djnos*) expression level (Fig. S3A). This result implies that the overall stem cell system (the sole cells with proliferative capability in planarians) is a selective target of the drug. To support this possibility,

the change in expression level of *Djinx-11*, a marker of committed progenitors, has been analyzed by real time RT-PCR. *Djinx-11* gene encodes a planarian stem cell gap junction protein required for the movement of small molecules that control maintenance, migration and differentiation of the progeny of proliferative cells (Oviedo and Levin, 2007). The expression level of this gene did not significantly change after chelidonine treatments (Fig. 4D-F). To evaluate possible cytotoxic side effects of this alkaloid to postmitotic cells, another member of the innexin gene family, *Djinx1*, whose expression is limited to the intestine, was examined (Oviedo and Levin, 2007). *Djsyt*, a planarian gene encoding synaptotagmin, a protein involved in the regulation of synaptic and extra-synaptic membrane trafficking in the central nervous system, was also used as a marker specific for nerve cells. Our data show that chelidonine treatments do not affect the expression level of these genes (Fig. S3B; Fig. 4G-I). On the whole, these results imply that chelidonine exposure causes no effects on differentiated cell types, such as intestinal or nerve cells.

3.3 Chelidonine, at a concentration of 20μM, prevents cell cycle progression of stem cells

To visualize in detail the effects of chelidonine on cell cycle and morphology of planarian cells, macerates (i.e. single cell types obtained by cell dissociation of worms: Baguñà and Romero, 1981) have been examined in specimens exposed to 20μM chelidonine and compared to macerates of control planarians treated with 0.1% DMSO. Macerates from planarian head and tail fragments (3 days of regeneration) were used in order to maximize the number of mitotic figures in the samples. Chelidonine treatment affected proliferating cells, resulting in abnormal metaphase and anaphase/telophase figures, as visualized by counterstaining with Hoechst 33342 in Fig. 5A-F. Treated interphase cells were unaffected (data not shown). To assess the possibility that cells exposed to

chelidonine do not progress in the cell cycle, the percentage of metaphase and anaphase/telophase figures has been compared in macerates obtained from both chelidonine-treated and DMSO control planarians. As shown in Fig. 5G the percentage of cycling cells, able to proceeds to anaphase/telophase, appeared significantly reduced in planarians treated with chelidonine with respect to the controls. Under the anti-proliferative effect of chelidonine, in fact, the percentage of metaphase figures, calculated by the analysis of the ratio between metaphases and anaphases/telophases in 0.1% DMSO controls and 20μM chelidonine, resulted significantly higher in the treated specimens that in the controls. The simplest explanation of these results is that a number of cycling cells remain arrested at metaphase.

4. Discussion

In this work, we demonstrate the anti-proliferative effect of chelidonine for the first time on stem cells *in vivo*, using planarians as an animal model. Chelidonine is one of the principal alkaloids obtained from *C. majus*, a plant with a broad spectrum of pharmacological activities. Although chelidonine lacks DNA-binding capacity, this alkaloid possesses significant anti-proliferative effects, and has the ability to arrest cell growth in different cell lines. These results support the possibility that chelidonine is an effective anticancer drug (Kaminskyy et al., 2008; Philchenkov et al., 2008). Different mechanisms have been proposed to explain the properties of this drug. Inhibition of telomerase activity and apoptosis stimulation has been described in primary human uveal melanoma cells **(**Kemény-Beke et al., 2006; Noureini and Wink, 2009). Literature data also demonstrate that chelidonine blocks mitosis by inhibition of tubulin polymerization and activation of the stress activated protein kinase/Jun kinase pathway (Panzer et al.,

2001; Wolff and Knipling, 1993). Although these effects have been proved in different cell lines, no data are available on stem cells *in vivo***.** Planarians are highly plastic animals (Gentile et al., 2011; Wagner et al., 2011). During regeneration planarian stem cells activate an intense proliferative program to restore the lost body parts. The presence of two distinct cellular compartments (a proliferative compartment and a functional, postmitotic compartment), as well as the use of cell-specific markers, makes regeneration a unique experimental model to predict drug-induced effects on single cell types. We have demonstrated that 20μM chelidonine treatment causes morphological defects in these animals, and also prevents regeneration, mimicking the phenotypic effects produced by irradiation or functional ablation of stem cell genes (Guo et al., 2006; Reddien et al., 2005). These results raise the possibility that chelidonine affects viability of stem cells. However, we observed that the chelidonine-induced effects were reversible, as most of the treated specimens recovered a normal morphology and regenerative capability after the transfer in water. This finding suggests that partial reduction in the number of stem cells occurred. Survived stem cells could then repopulate the body of the animals in a short time (Salvetti et al., 2009, Wagner et al., 2011). Our results support the possibility that this alkaloid blocks cell proliferation with limited cytotoxic effects (Kaminskyy et al., 2008). Although precise calibration of a drug concentration *in vivo* is difficult to achieve, especially when administered by soaking, we observed that the anti-proliferative effects of chelidonine depended on the applied dose. Anti-proliferative drugs are usually administered *in vivo* at a concentration required to completely halt the growth of target cells (total growth inhibition: TGI). The most effective treatment on planarians was 20μM chelidonine. This value is comparable to chelidonine TGI on several tumor cell lines. A wide screening on 60 different tumor cell lines performed at NIH shows, in fact, a TGI for chelidonine of 20μM (Developmental Therapeutics Program NCI/NIH, website

http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp, searching for "chelidonine" and selecting NCI60 Cancer Screen Current Data).

In order to develop chelidonine to its full potential clinical applications, it is pivotal to obtain a clearer understanding of the molecular mechanisms that underlie its effects on stem cells *in vivo*. Because these studies are difficult in mammalian models, planarians and their pluripotent stem cells represent a suitable complementary system for dissecting relevant molecular details. We have followed a molecular strategy to analyze and link variation of gene expression with drug response of specific cell types. The use of molecular markers that characterize proliferating cells demonstrates that 20μM chelidonine provokes a significant diminution in the expression level of these genes. Lack of side effects produced by DMSO long-term exposure has been proved by whole mount *in situ* hybridization and real time RT-PCR on regenerating planarians exposed to different DMSO concentrations (0.025%-0.1%) or maintained in water. On the whole the results demonstrate that DMSO i) does not cause morphological defects, ii) does not interfere with regenerative capability or with *Djmcm2* expression and prove that the observed dose-effect is related to an increase in chelidonine concentrations and not in DMSO concentrations, suggesting that the effects of DMSO and chelidonine are not cumulative. It is important to point out that the effect of the drug extends to different stem cell subpopulations, as demonstrated by the significant decrease of the *Djnos*-positive germline stem cells in chelidonine-treated animals. These results provide evidence that 20μM dose reduces significantly cell growth and affects germline stem cell viability. No effects were detected using markers generally employed to characterize differentiated cell types, such as nerve or intestinal cells, suggesting that no generalized cytotoxic effect occurred. These results represent the first gene expression analysis in relation to chelidonine activity *in vivo* and reveal that the drug specifically affects dividing stem

cells, whereas postmitotic cells are not influenced.

We sought to understand how chelidonine interferes with the planarian stem cell system. Our data demonstrate that the effects of chelidonine on cell cycle progression may depend on inhibition of tubulin polymerization. A significant arrest of cells in the mitotic metaphase was observed as a consequence of chelidonine treatment. The inhibitory effect on dividing cells resulted in fact in abnormal accumulation of metaphase figures, while the incidence of anaphases/telophases consistently decreased. In addition, numerous anomalous mitotic cell forms could be observed. A chelidonine-mediated partial inactivation of the mitotic spindle, with consequent cytostatic effects on root tip cells (i.e. cells with stem-cell-like properties), has been described in *Allium cepa* (Krahulcová, 1979). It is possible that this alkaloid acts at the colchicine-binding site, as it is a weak competitive inhibitor of colchicine binding (Wolff and Knipling, 1993). Even though these studies varied in methodology and sample, the results are remarkably consistent with our results, suggesting that the inhibitory effect of chelidonine on tubulin polymerization represents a major mechanism for growth arrest of dividing cells *in vivo*. The results of this research, according with previous studies on cell lines, support the idea that chelidonine represents an important anti-proliferative alkaloid devoid of significant cytotoxicity and emphasize the potential value of planarian stem cells in finding better drugs to treat human disease.

5. Conclusions

To the best of our knowledge, this study is the first to report the effects of chelidonine on arrest of cell cycle in stem cells *in vivo*. Planarian model is also a rich source of information about the mechanisms of cell growth inhibition. Based on our data we

hypothesize a mechanistic scenario that predicts how inhibition of tubulin polymerization plays a key role in the function of this drug. These findings provide new information to complete the understanding of the chelidonine anti-proliferative effects, and support the possible potential of this drug for the therapy of hyper-proliferative disorders, such as cancer and psoriasis.

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Figure legends

Figure 1. Schematic of amputation procedure of a planarian. Black line indicates the amputation level. Complete regeneration of the missing body parts occurs in about 10 days at 18°C. Regenerated body parts are shown in light grey. ph: pharynx.

Figure 2. Brightfield images of planarians after 20μM chelidonine treatment. (A) Schematic representation of an intact planarian. (B) An intact control planarian 0.1% DMSO. (C) An intact planarian after 3 days of chelidonine treatment shows body lesions. (D) An intact planarian after 3 days of chelidonine treatment shows ventral curling. (E) Schematic representation of a planarian tail fragment regenerating a head. (F) A 0.1% DMSO control tail fragment regenerating a head, 3 days after amputation. (G) A tail fragment treated with chelidonine did not form regeneration blastema, 3 days after amputation. (H) A tail fragment treated with chelidonine shows a very reduced blastema with blisters, 3 days after amputation. Anterior is up. Dashed line in D and E corresponds to the original amputation site. Scale bar: 1 mm.

Figure 3. *In situ* hybridization with *Djmcm2* of planarian head fragments after 20μM chelidonine treatment. (A) Schematic representation of a planarian head fragment regenerating a tail. (B) A 0.1% DMSO control regenerating fragment shows strong hybridization signal, visualized as brown color by Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) chromogen precipitation in the mesenchymal tissue, where planarian stem cells are located. (C) A comparable fragment hybridized after chelidonine treatment shows reduced *Djmcm2* expression. Six days of regeneration. Anterior is up. Scale bar: 0.5 mm.

Figure 4. Expression level of *Djmcm2*, *Djinx-11* and *Djsyt* analyzed in planarians after chelidonine treatment by real time RT- PCR. (A-C) *Djmcm2* expression level**.** **(**A) 0.025% DMSO controls (DMSO) and 5μM chelidonine (CH). (B) 0.05% DMSO controls (DMSO) and 10μM chelidonine (CH). (C) 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). (D-F) *Djinx-11* expression level. **(**D) 0.025% DMSO controls (DMSO) and 5μM chelidonine (CH). (E) 0.05% DMSO controls (DMSO) and 10μM chelidonine (CH). (F) 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). (G-I) *Djsyt* expression level. **(**G) 0.025% DMSO controls (DMSO) and 5μM chelidonine (CH). (H) 0.05% DMSO controls (DMSO) and 10μM chelidonine (CH). (I) 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). Expression levels are indicated in relative units, assuming a value of 1 for DMSO-treated controls. Samples were compared using the Kruskal-Wallis non-parametric ANOVA. **P<0.01.

Figure 5: Mitotic abnormalities arise *in vivo* in response to 20μM chelidonine treatment.

(A-C) Mitotic figures after 0.1% DMSO treatment (DMSO). (D-F) Mitotic figures after 20μM chelidonine treatment (CH). (A**)** Metaphase chromosomes appear arranged in the middle of the cell on the metaphase plate before being separated into each of the two daughter cells at the anaphase stage. (B) A cell at anaphase, usually defined as the separation of the sister chromatids by shortening of the microtubules of the mitotic spindle. (C) A telophase figure: both sets of chromosomes become now surrounded by new nuclei and unfold back into chromatin, while cytokinesis completes cell division. (D) A metaphase figure after chelidonine treatment. The chromosomes appear abnormally arranged in the cell. (E) An anaphase figure after chelidonine treatment, showing altered

chromosome separation. (F) A telophase figure after chelidonine treatment. In this cell cytokinesis did not occur to separate the daughter cells, resulting in a cell containing two condensed groups of chromosomes. Scale bar: 10 μm. (G) Analysis of the ratio between metaphases and anaphases/telophases in 0.1% DMSO controls (DMSO) and 20μM chelidonine (CH). Values are expressed as percentages with respect to controls, to which the arbitrary value of 100% has been assigned. Each value represents the mean ± standard deviation of six independent samples counted in duplicate. Samples were compared using t- test. **P<0.001.

Supporting information

Figure S1: **Expression levels of *DjEF2* and *DjACTB* reference genes after DMSO treatment, analyzed by real time RT-PCR.** (A) *DjACTB*. (B) *DjEF2*. Expression levels are indicated in relative units, assuming a value of 1 for water-treated controls.

Figure S2. Effect of different DMSO concentrations, compared to water as a negative control. (A,B) *Djmcm2* expression as visualized by situ hybridization of tail fragments regenerating a head, 6 days of regeneration. (A) A fragment maintained in water. (B) A fragment in 0.1% DMSO. Comparison of the obtained results demonstrates that both fragments regenerate normal heads and show similar *Djmcm2* expression. Dashed line in A and B corresponds to the original amputation site. (C) Expression level of *Djmcm2* compared by real time RT-PCR in regenerating planarians (3 days of regeneration), exposed to different DMSO concentrations (0.025%-0.1%) or maintained in water. Expression levels are indicated in relative units, assuming a value of 1 for water-treated controls.

Figure S3: **Expression level of *Djnos* and *Djinx1* analyzed in planarians after 20μM chelidonine treatment by real time RT-PCR.** (A) *Djnos* expression level in chelidonine-treated planarians (CH) and 0.1%DMSO controls. (B) *Djinx1* expression level in chelidonine-treated planarians (CH) and DMSO 0.1%controls. Expression levels are indicated in relative units, assuming a value of 1 for DMSO-treated controls. Samples were compared using the Kruskal-Wallis non-parametric ANOVA. *P<0.05.

Video S1: A representative example of a ‘curled’ planarian fragment, as produced by 20μM chelidonine treatment. A small blastema, with a blister, is barely visible at the anterior region, 3 days after amputation (600 frames, 66.7 ms per frame).

Video S2: A representative example of a 0.1% DMSO regenerating control planarian is shown for comparison. Blastema is visible as a white area at the anterior region, 3 days after amputation (510 frames, 66.7 ms per frame).