Comparative Biochemistry and Physiology, Part B 253 (2021) 110550

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Humoral responses during wound healing in *Holothuria tubulosa* (Gmelin, 1788)

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ARTICLE INFO

Keywords:
Cytotoxic activity
Echinoderm
Enzyme activity
Regeneration mechanism
Immunity sea cucumber

ABSTRACT

Wounds in living organisms trigger tissue-repair mechanisms. The sea cucumber (*Holoturia tubulosa*) is an excellent model species for achieving a better understanding of the humoral and cellular aspects involved in such healing processes. Consequently, this study assesses data on its morphometric, physiological and humoral responses 1, 2, 6, 24 and 48h after wound induction. In particular, morphometric data on the weight, width, length and coelomic-fluid volume of the species were estimated at different times during our experiments. In addition, the humoral aspects related to the enzymatic activity of esterase, alkaline phosphatase and peroxidase, as well as the cytotoxic activity of cell lysates (CL) and cell-free coelomic fluids (CfCf) are evaluated for the first time. Our results reveal a significant decrease in body length and weight, along with time-dependent, significant changes in the esterase, alkaline phosphatase, peroxidase and cytotoxic activity in both the CL and CfCf. The data obtained lead to the pioneering finding that there is an important time-dependent involvement of morphometric (changes in weight and length) and humoral (enzymatic and cytotoxic) responses in wound healing.

1. Introduction

A variety of marine organisms are important resources for bioactive molecules and are able to implement a diverse range of immune responses when exposed to different stressful conditions (Cordero et al., 2016; Inguglia et al., 2020; Mauro et al., 2020a, 2020b; Chiaramonte et al., 2020; Vazzana et al., 2020a, b). Repair and regeneration processes come into play in all living organisms as a consequence of wound stress, enabling the restoration of damaged tissue and organs. These processes are known as *epimorphosis* and *morphallaxis*, and have been studied over the years in various invertebrates (Mladenov et al., 1989; Moss et al., 1998; Agata et al., 2007; Biressi et al., 2010; Vazzana et al., 2015; de Jong and Seaver, 2016; Kostyuchenko and Kozin, 2020). These species have proved to be important models for understanding not only different human diseases (Thompson and Marsh, 2003; Mhatre et al., 2013; Mauro et al., 2020b; Luparello et al. 2020a), but also how tissue and organs are generated (Allen et al., 2001; Candia Carnevali and Bonasoro, 2001a; Rychel and Swalla, 2009; Licciano et al., 2012; Khadra et al., 2018; Nikanorova et al., 2020). Echinoderms in particular have played a prominent role in developing our understanding of human disease and tissue-regeneration processes (Dupont and Thorndyke, 2007; García-Arrarás and Dolmatov, 2010; Lazzara et al., 2019; Luparello et al., 2020b).

Regeneration is an important aspect of the life of echinoderms, with significant biological implications for the survival of individuals and the colonization of new environments (Candia Carnevali, 2006). An example is the extraordinary ability of starfish to fully regenerate their arms after amputation (Mladenov et al., 1989; Moss et al., 1998), or to completely re-epithelialize their body surface after injury (Candia Carnevali and Bonasoro, 2001b; Biressi et al., 2010; Thorndyke et al., 2001).

Even the Holothuroidea class (sea cucumbers) has an exceptional and unique capacity to regenerate the digestive system and associated organs after evisceration (Garcia-Arraras et al., 2001; Dolmatov et al., 2012). Rapid and efficient repair processes are important for preventing the loss of coelomic fluid, as well as subsequent invasions of external pathogens (Moss et al., 1998; Dolmatov et al., 2012). Several studies have highlighted that the immune mechanisms, both cellular and humoral, are involved in wound regeneration (Garcia-Arraras et al., 2006; San Miguel-Ruiz and Garcia-Arraras, 2007). Coelomocytes play very

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https://doi.org/10.1016/j.cbpb.2020.110550
Received 13 July 2020; Received in revised form 14 December 2020; Accepted 18 December 2020
Available online 23 December 2020

important immune-related roles (Canicattì, 1990; Pagliara et al., 2003; Sun et al., 2008; Vazzana et al., 2020a; Chiaramonte et al., 2020). Indeed, in *Holothuria glaberrima*, these cells have been shown to increase in number after injury to enable wound coagulation processes (San Miguel-Ruiz and Garcia-Arraras, 2007). For *Holothuria polii*, similar findings have been made by Canicattì and Farina-Lipari (1990); Canicatti et al. (1992), who demonstrated the relevant roles of spherulocytes and amebocytes in the early stages of coagulation. In the case of *H. tubulosa*, Vazzana et al. (2015) observed that these organisms respond to cut stresses with significant changes in: the number of circulating coelomocytes; the expression levels of heat-shock proteins (HSPs) in coelomocytes, scar tissue and cell-free coelomic fluid (CfCf); and total protein concentrations.

As a result of stressful conditions, immune mechanisms like the activities of enzymes and cytotoxins play an important part in the restoration of homeostasis (Parisi et al., 2017; Vazzana et al., 2015, 2018; Vazzana et al., 2020a, b). When it comes to the actions of enzymes, the most studied are those belonging to the hydrolase class, including alkaline phosphatase and esterase, which play a key role in the removal of phosphate groups (Xue and Renault, 2000) and ester bonds (Hannam et al., 2008), respectively. They are also involved in detoxification and inflammation processes via their involvement in the response to various stress conditions (Parisi et al., 2017; Vazzana et al., 2020a, b). Like the hydrolase-class enzymes, antioxidant versions such as peroxidase have a crucial role in the restoration of homeostasis in invertebrates (Mydlarz and Harvell, 2007). The actions of these enzymes reduce oxidative stress by lowering the levels of reactive oxygen species (ROS) in circulation, thereby restoring homeostasis (Livingstone, 2003). Cytotoxic activity also play an important part in the response to stressful conditions (Parrinello et al., 1979), acting through lysins that can occur naturally in bodily fluids or are secreted by coelomic cells (Arizza et al., 2007; Vazzana et al., 2018).

Despite our knowledge of the important role played by these biomarkers in the effects of stress on invertebrates, there have been no studies to date that evaluate their possible involvement in the immune response of echinoderms during a wound-healing event. Even though echinoderms are known to have extraordinary regenerative capacities, these have not yet been explored in a way that improves what is understood of the repair and regeneration phase in vertebrates. Moreover, to the best of our knowledge, only the work in the older literature describes any involvement of enzymes from the hydrolase class in wound-healing and regeneration processes (Junqueira, 1950; Raekallio and Levonen, 1963; Betz, 1994). In particular, identification of the high concentrations of alkaline phosphatase in the fibroblasts of the regenerating tails of the tadpole (Junqueira, 1950) has led to the finding that it participates in the growth and regeneration of tissue. It has also been observed that esterase activity tends to increase during the healing processes of human skin (Betz, 1994), and that the rise in the number of fibroblasts changes the enzyme's function during phagocytosis (Raekallio and Levonen, 1963). Being that the activities of the enzymes belonging to the hydrolase classes play a key role in wound healing, any therapy that normally only acts to prevent pathogens could be oriented towards influencing the activity of these hydrolase enzymes (Malik, 1971).

Our research aims to evaluate the wound-healing processes in *H. tubulosa*, in particular the involvement of the cytotoxins and enzymes (esterase, alkaline phosphatase and peroxidase) present in the coelomic fluid and coelomocytes. This includes an investigation of the changes in the animals' length, width and weight, as well as the total volume of their coelomic fluid.

2. Materials and methods

2.1. Animals

Seventy-two *H. tubulosa* individuals (mean±SD, weight 60±19 g; length 8±0.99 cm) were collected from their natural environment, in our case off the coast of Santa Flavia (Palermo, Tyrrhenian Sea). They were then acclimated in the aquarium of the STEBICEF department of the University of Palermo for two weeks. In particular, they were placed in a rectangular tank (100 cm x 50 cm x 50 cm) containing 250 L of continuously oxygenated natural seawater set at a temperature of 15±2 °C. The individuals were fed using commercial invertebrate pellets (Azoo, Taikong Corp. Taiwan), but received no food in the 24h before the start of the experiments.

2.2. Experiment design

After the acclimatization period, each individual was taken from the tank, allowed to initially contract and expel water (due to handling), and then weighed using an electronic balance. The length and width were measured with an ictiometer and a calliper, respectively. Thereafter, a sterile scalpel was employed to create a "wound" by making a 1 cm incision on the ventral anterior part of all the animals other than those used as the "control" sample. The specimens were then returned to the tank, where the coelomic fluid was sampled through a different cut in the dorsal part of the body at 0h (control: hereafter T0), 1h (T1h), 2h (T2h), 6 h (T6h), 24h (T24h) and 48h (T48h). We sampled 12 of the 72 individuals for each of the six treatments (five experimental and one control). Each animal was sampled only once.

2.3. Biological samples

The coelomic fluid was sampled in a graduated test tube using an ISO-EDTA anticoagulant solution (0.5M NaCl, 20mM Tris-HCl, 30mM EDTA; pH 7.4) (6:1), which enabled us to measure the total fluid volume taken from each animal. All the samples were then centrifuged at 400 g for 10 min at 4 °C to produce a cellular pellet and the Cell-free Coelomic fluid (CfCf). Each pellet was: homogenized using a potter for 5 min in 250 μl of RIPA-buffer lysis 1X (Sigma-Aldrich) that contained a cocktail of 1:200 anti-protease; sonicated for 2 min; and centrifuged at 13,000 g for 10 min at 4 °C. The protein concentrations of the CfCf and the cell lysate (CL) samples were quantified according to Bradford's method (1976) and used to evaluate the activities of the enzymes (esterase, phosphatase and peroxidase).

2.4. Enzymatic assays: esterase, alkaline phosphatase and peroxidase

The enzymatic activities of the alkaline phosphatase and esterase in the CfCf and CL samples were evaluated by incubating 25 μl of the sample with 25 μl of buffer in 96-well plates (Ross et al., 2000). The buffer for the esterase activity was prepared using 0.4 mM of p-nitrophenyl myristate (Sigma-Aldrich) in a solution of 100 mM ammonium bicarbonate and 0.5% Triton X-100 (pH 7.8); for the alkaline phosphatase activity, the buffer was produced using 4 mM of p-nitrophenyl phosphate (Sigma-Aldrich) in a solution of 100 mM ammonium bicarbonate and 1 mM of $MgCl_2$ (pH 7.8). The enzymatic activity in each sample was measured three times every 5 min for 1h using a spectrophotometer (GloMax®-Multi Detection System; Promega Corporation, Madison, Wisconsin, USA).

The enzymatic activities were expressed in U/μg and calculated using the formula:

$$\{(Abs/min) \times (1000/Eb) \times (Vf/Vi)\},$$

in which: *Abs/min* is the absorbance value obtained for each sample divided by the time at which the measurement was taken (60 min); *Vf* indicates the final volume of the plate well; *Vi* represents the initial volume of the plate well; and *Eb* is an experimental constant (16.4 and 18.4 for the esterase and alkaline phosphatase activities, respectively). The U indicates the quantity of enzyme required to release 1 millimole of p-nitrophenol for 60 sec.

The enzymatic activity of the peroxidase was measured using the modified protocol of Quade and Roth, (1997). In detail, 25 μl of each

sample were incubated with 50μl of TMB solution (3.3', 5.5' tetramethylbenzidine) (TMB, Sigma, Italy) for 30 min in 96-well plates. Subsequently, the reaction was brought to an end by adding 50 μl of sulfuric acid 2 M to each sample, with the absorbance measured at 450 nm. The peroxidase activity was determined three times for each sample and expressed as U/μg of protein.

2.5. Cytotoxic activity assay

The cytotoxic activity in the CfCf and CL was evaluated by incubating each sample with sheep and rabbit erythrocytes supplied by the Experimental Zooprophylactic Institute of Sicily, A. Mirri. The erythrocytes were washed in a PBS solution (6 mM KH_2PO_4, 30 mM Na_2HPO_4, 0.11 M NaCl) and resuspended at 1% (8×10^6 fresh erythrocytes) in isosmotic media with calcio (ISO-Ca^{2+}, 0.5 M NaCl, 20 mM Tris-HCl, 10 mM $CaCl_2$; pH 7.4). Subsequently, 100 μl of each sample were incubated with 100 μl of erythrocytes at 37 °C for 60 min. The samples were then centrifuged at 1650 rpm for 10 min at a temperature of 4 °C. The amount of haemoglobin released was measured at 540 nm using a spectrophotometer (GloMax®-Multi Detection System; Promega Corporation, Madison, Wisconsin, USA). The following formula was employed to calculate the degree of haemolysis:

$$\frac{(Absmeasuredrelease - Absspontaneousrelease)}{(Abscompleterelease - Absspontaneousrelease)} \times 100$$

in which: *Abs measured release* is the absorbance value determined for each sample; *Abs spontaneous release* is the absorbance value measured by incubating only the erythrocytes with ISO-Ca^{2+}; and *Abs complete release* is the absorbance value relating to the total haemolysis, which was obtained by incubating only the erythrocytes and subsequently resuspending them in distilled water.

2.6. Statistical analysis

Given the inter-individual size variability, we conducted Kruskal Wallis ANOVA tests before starting the experiments. These revealed no divergences between the length (H 5.72 = 1.66; P = 0.894) and the weight (H 5.72= 3.42; P = 0.635) of the six median treatments, meaning that the inter-individual variability of these biometric measures would not introduce any bias into the differences recorded at the end of the trials.

After completing the experiments, we then tested whether, and when, the wounding event provoked changes in the selected body parameters (i.e., length, width, weight, total volume of coelomic fluid) and the enzymatic (i.e., esterase, alkaline phosphatase, peroxidase) and cytotoxic activity (i.e., in both the rabbit and sheep erythrocytes). The outcomes for each variable other than the body parameters were determined in both the CL and CfCf samples, bringing to 14 the total number of variables analyzed (see Table 1 for a systematic list). A non-parametric test was employed because our data was not normally distributed. All 14 variables were treated as dependant in the Kruskal-Wallis ANOVAs, where the time from the wound-healing event was regarded as an independent factor with six levels (T0: control, T1h, T2h, T6h, T24h and T48h). The descriptive indices for all the variables are expressed as the mean ± standard deviation (SD). The STATISTICA 10 StatSoft Inc. package (1984-2011) was used for all of the tests conducted.

Table 1
Kruskal-Wallis ANOVA results showing the effects at six post-wounding times on each variable measured in the *H. tubulosa* specimens ($N = 72$).

Variables	H	*p*
Lenghts	37.67	<0.0001
Width	2.17	0.8250
Weight	13.58	0.0185
Coelomic fluid volume	15.52	0.0084
Esterase (CL)	33.83	<0.0001
Esterase (CfCf)	43.44	<0.0001
Alkaline phosphatase (CL)	39.62	<0.0001
Alkaline phosphatase (CfCf)	40.46	<0.0001
Peroxidase (CL)	36.62	<0.0001
Peroxidase (CfCf)	44.54	<0.0001
Cytotoxicity against rabbit erythrocytes (CL)	37.05	<0.0001
Cytotoxicity against rabbit erythrocytes (CfCf)	30.01	<0.0001
Cytotoxicity against sheep erythrocytes (CL)	40.65	<0.0001
Cytotoxicity against sheep erythrocytes (CfCf)	23.87	0.0002

3. Results

3.1. Morphometric data and volume of coelomic fluid

Only the body length and weight of the *H. tubulosa* individuals used in our experiments changed significantly (Table 1; Fig. 1). No significant differences were observed with respect to width (although it varied within a small range) or the total volume of the coelomic fluid. In relation to the latter, this was despite an indication of significantly different changes across the experiments (Table 1), with postdoc tests revealing non-significant variations from one trial to another (Table 2; Fig. 1D). In terms of the post-wounding length, significant reductions of 34% were observed 6h (5.8±0.407 cm) after the cutting event compared to the controls (8.8±0.769 cm, post-hoc Kruskal-Wallis ANOVA tests; Table 2), with a subsequent recovery already observable at 24 h (7.0±1.803 cm) (Fig. 1A). The weight at 6h decreased significantly, although the high response variability demonstrated by an adequate range of SDs indicated a reduced test power (Fig. 1C).

3.2. Enzymatic activity in the CL and CfCf samples

Overall, there were significant changes in the enzymatic activity in both the CL and CfCf samples (Table 1; Figs. 2 and 3). More specifically, significant increases in the levels of esterase and alkaline phosphatase were observed in the CL samples 1 h after the wounding event (Table 2; Fig. 2A and 2C); meanwhile, significant increases were also observed in the CfCf samples after 24 h for both enzymes (Table 2; Fig. 2B and 2D). Compared to the control specimens (0.022±0.014 U/μg), there was a significant, four-fold increase (Table 2) in esterase activity in the CL samples just 1 h after the cut was made (0.092±0.022 U/μg). This value then returned to that of the control group after 2h (0.022±0.001 U/μg), and remained unchanged until 48 h had elapsed (0.017±0.008 U/μg) (Table 2; Fig. 2A). In the CfCf samples, although the esterase activity showed significant increases 2 and 6 h after the wounding (Table 2), this was not palpable given the high variability of the data (Table 2; Fig. 2B). The activity level returned to that observed in the control sample after 48 h (0.007±0.003 U/μg) (Table 2; Fig. 2B).

The alkaline phosphatase activity in the CL samples followed the same trend as the esterase (Fig. 2C); there was a significant, three-fold increase (Table 2) 1h after the cut was made (0.185±0.017 U/μg; Table 2) compared to the control (0.068±0.034 U/μg; Table 2; Fig. 2C). The activity returned to the levels of the control group 48h later (0.058±0.025 U/μg). Compared to the controls (0.004±0.001 U/μg; Fig. 2D), the activity in the CfCf samples was significantly higher (Table 2), increasing three-fold 24h after the wounding (0.011±0.000 U/μg; Table 2) (0.004±0.001 U/μg; Fig. 2D). These values returned to levels similar to those of the controls 48h later (0.004±0.001 U/μg).

Compared to the control group (37.595±10.997 U/μg) (Table 2; Fig. 3A), there was a significant, two-fold decrease in the peroxidase activity in the CL samples 1h after the cutting event (17.938±6.442 U/μg). Thereafter, this activity was consistently and significantly lower (Table 2) after 48h (15.210±1.866 U/μg; Fig. 3A). Assessed against the controls (16.504±4.483 U/μg), the peroxidase activity in the CfCf samples showed significant, three-fold increases (Table 2) just 1h after the cut was made (43.814±2.765 U/μg). However, this activity dropped after 2h (13.824±4.055 U/μg), becoming similar to that of the controls;

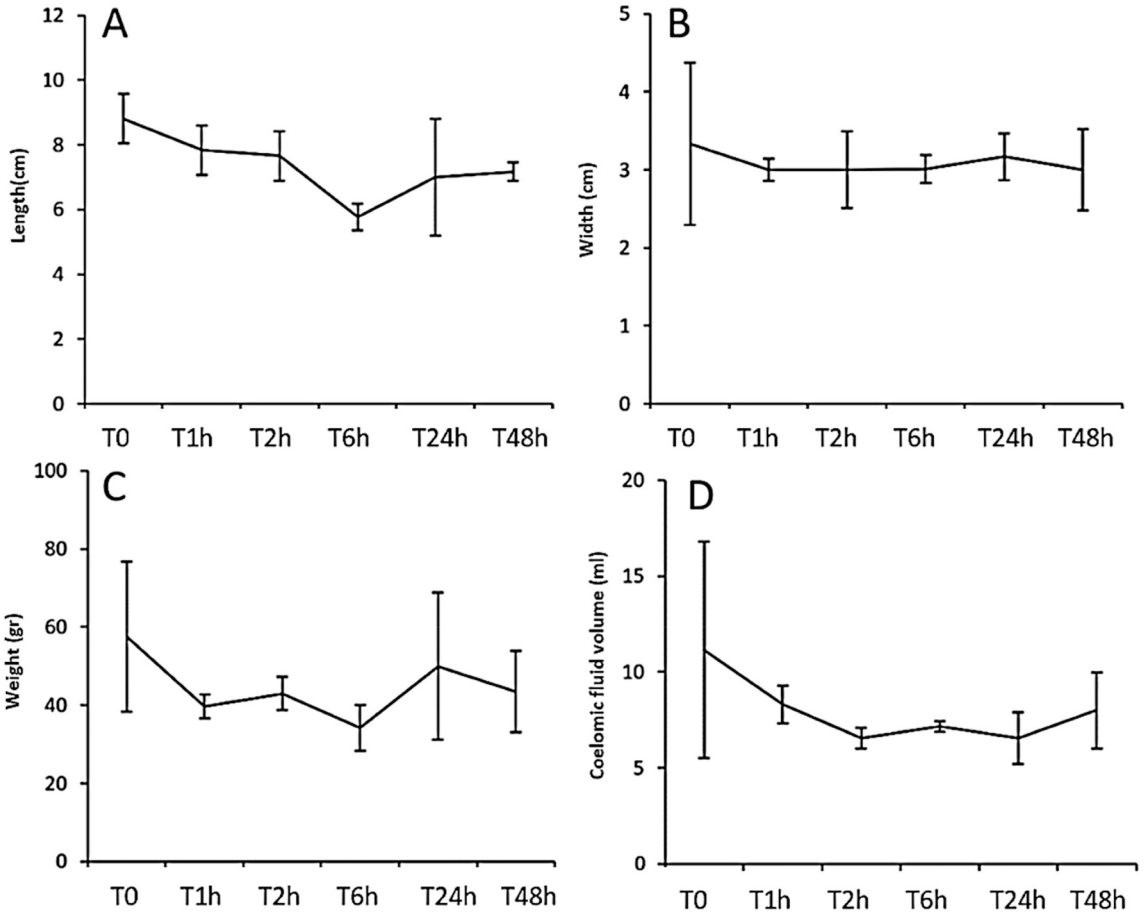

Fig. 1. A) Length, B) Width, C) Weight, and D) Total coelomic fluid volume of the *H. tubulosa* specimens, measured at different post-wounding times (from T1h to T48h) and in the controls (T0). The values in the graphs are shown as the mean ± SD.

Table 2
Kruskal-Wallis ANOVA post-hoc tests showing the P values of the comparisons between the values recorded at each post-wounding time (T1h-T48h) and those measured at the control time (T0; i.e., the variable measured at the precise moment of the wounding event).

Variables	T1h	T2h	T6h	T24h	T48h
Lenghts	1.0000	0.3502	**<0.0001**	**0.0067**	**0.0111**
Width	1.0000	1.0000	1.0000	1.0000	1.0000
Weight	1.0000	1.0000	**0.0093**	1.0000	1.0000
Coelomic fluid volume	1.0000	1.0000	1.0000	1.0000	1.0000
Esterase (CL)	**0.0011**	1.0000	1.0000	1.0000	1.0000
Esterase (CfCf)	1.0000	**0.0426**	**0.0075**	**<0.0001**	1.0000
Alkaline phosphatase (CL)	**0.0115**	1.0000	1.0000	0.2154	1.0000
Alkaline phosphatase (CfCf)	1.0000	1.0000	1.0000	**<0.0001**	1.0000
Peroxidase (CL)	**0.0014**	1.0000	0.0584	1.0000	**<0.0001**
Peroxidase (CfCf)	**0.0002**	1.0000	1.0000	1.0000	1.0000
Cytotoxicity against rabbit erythrocytes (CL)	**0.0027**	**<0.0001**	**0.0033**	**<0.0001**	0.6834
Cytotoxicity against rabbit erythrocytes (CfCf)	0.1462	1.0000	1.0000	**<0.0001**	1.0000
Cytotoxicity against sheep erythrocytes (CL)	**0.0044**	**0.0168**	1.0000	0.1730	1.0000
Cytotoxicity against sheep erythrocytes (CfCf)	1.0000	1.0000	1.0000	**0.0319**	1.0000

it remained constant until 48h later (Fig. 3B).

3.3. Cytotoxic activity in the CL and CfCf samples

The cytotoxic activity of the CL and CfCf samples was evaluated using rabbit and sheep erythrocytes, with both showing significant changes (Table 1; Fig. 4). Compared to the control sample (17.5 ± 0.32%; Fig. 4A), the treatment with the rabbit erythrocytes increased the cytotoxic activity of the CL significantly (Table 2) at all post-injury times other than 48 h (18.55 ± 0.58% (T1h); 19.02 ± 0.71% (T2h); 18.62 ± 0.02% (T6h); 19.01 ± 1.11% (T24h)). Again, compared to the controls (12.65 ± 1.09%; Fig. 4C), the treatment with the sheep erythrocytes increased the cytotoxic activity of the CL samples significantly (Table 2) 1h (14.86 ± 1, 27%) and 2h (14.51 ± 0.74%) after the injury. On the other hand, compared to the controls (13.92±0.03%; 18.92 ± 7.42%), the increased cytotoxic activity in the CfCf samples was significant (Table 2) only 24 h after treatment with both the rabbit (29.21 ± 2.00%, Fig. 4B) and sheep erythrocytes (27.58 ± 2.17%, Fig. 4D). This is despite the high variability of the SDs.

4. Discussion

4.1. Morphometric data

Holothurias are known to be soft-bodied echinoderms and, for this reason, can stretch or contract using locomotion and feeding, respiration and sensory functions (Barnes et al., 1990). This makes their length, width and body weight parameters highly variable. As reported by Pitt and Duy (2004), obtaining (more) accurate average sizes requires

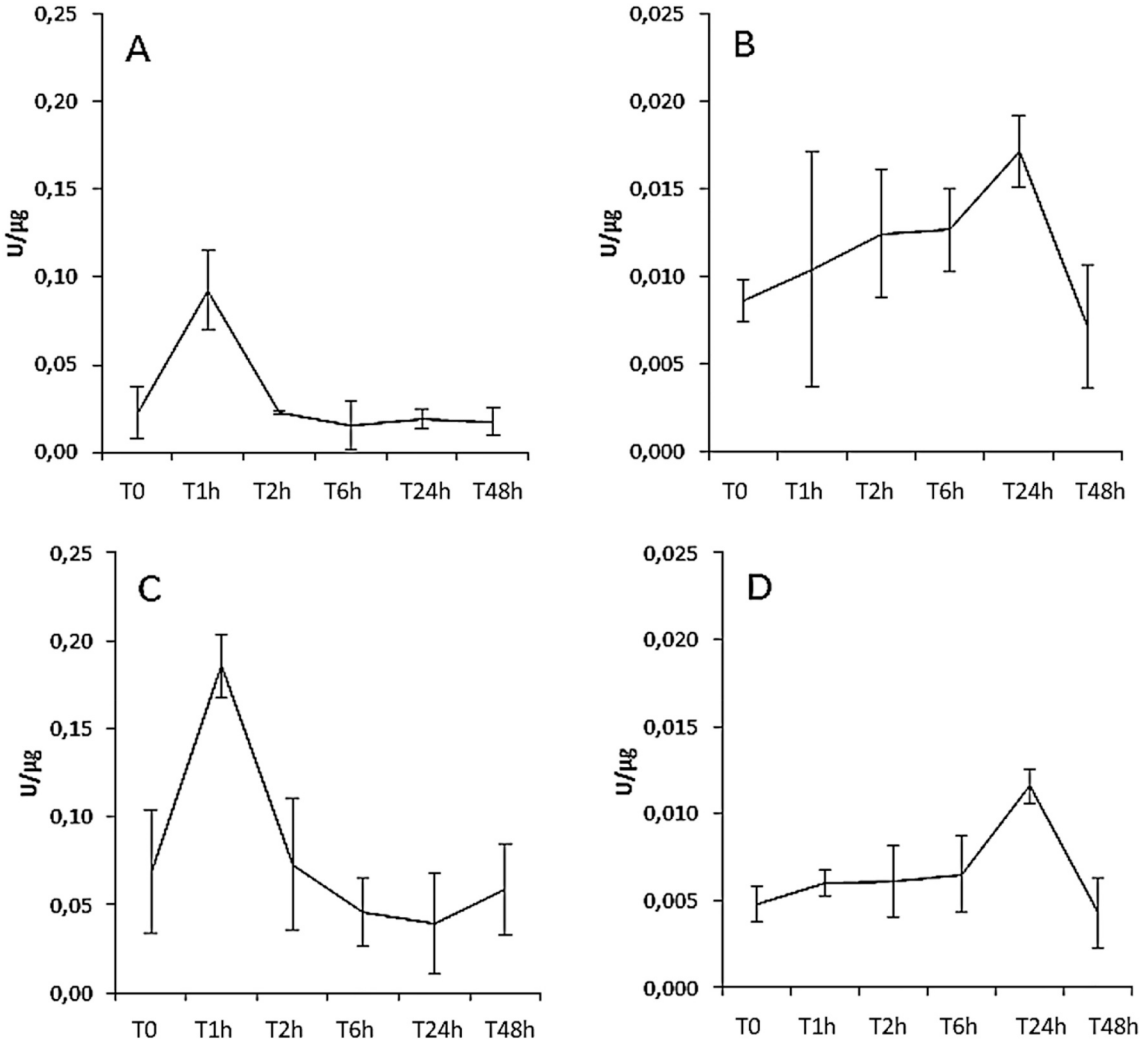

Fig. 2. A), B) Esterase activity of the CL and CfCf samples; C), D) Alkaline phosphatase activity of the CL and CfCf samples taken from the *H. tubulosa* specimens, measured at different post-wounding times (from T1h to T48h) and in the controls (T0). The values are expressed as the mean ± SD.

changes in a group of animals to be tracked over a few days by measuring their length and weight at fixed intervals. This paper shows for the first time how variations in these morphometric data can have significant correlations with injury stress. In fact, we observed changes in the length and weight of our adult *H. tubulosa* specimens that depended on the time elapsed after the wounding event.

In particular, there was a decrease in the total volume of coelomic fluid 1 h after a cut was made. Although not significant (probably due to high data variability, especially at T0; Fig. 1D), these reductions consistently led to major decreases in the weight and length of the specimens 6h after the wounding. It is possible that the animals begin to contract by reducing both their weight and length in an attempt to reduce fluid loss. These morphometric changes may, therefore, have depended on the loss of coelomic fluid through the wound inflicted at the start of the experiments. Furthermore, at 48 h, there had been some, albeit not significant, recovery of the total volumes of coelomic fluid. This concurs with the finding by Vazzana et al. (2015), whose research also noted the initiation of the wound-healing process. This confirms that the changes in the morphometric data identified in our study were due to shear stress and, therefore, the loss of coelomic fluid. In fact, it has been reported that coelomic fluid is important in the stress response of echinoderms (Buchanan, 1969; Binyon, 1972; Wahltinez et al., 2020), and can have a negative effect on behavioural and physiological responses (Dolmatov et al., 2012). In sea cucumbers, the fluid fills the coelomic cavity, surrounds the internal organs, and provides information on systemic biochemical and cellular processes, as well as potential stress-response mechanisms for restoring homeostasis (Collard et al., 2013; Wahltinez et al., 2020).

4.2. Enzyme activity in CL and CfCf

In Holothuroids, the coelomic fluid volume constitutes a significant fraction of their body mass, and the coelomocytes that form the cellular component responsible for innate cell-mediated immunity are considered to be functionally equivalent to the leukocytes of vertebrates. In addition to its cellular element, coelomic fluid also consists of CfCf, which contains soluble factors secreted constitutively by different parts of the body, even in particular physiological conditions. This provides an opportunity to develop a general profile of the biological state of the organism. Numerous soluble factors are directly or indirectly involved in a variety of physiological processes such as wound healing, inflammation and tissue remodelling. Indeed, it is for these reasons that they are released into extracellular body fluids, where they act as biomarkers of their corresponding processes (Shabelnikov et al., 2019). These factors include enzymes that play an important bactericidal humoral role in the defence against pathogens or other foreign substances.

In our study, we evaluated changes in the enzyme activities of the hydrolase class. It is known from the literature that these enzymes play

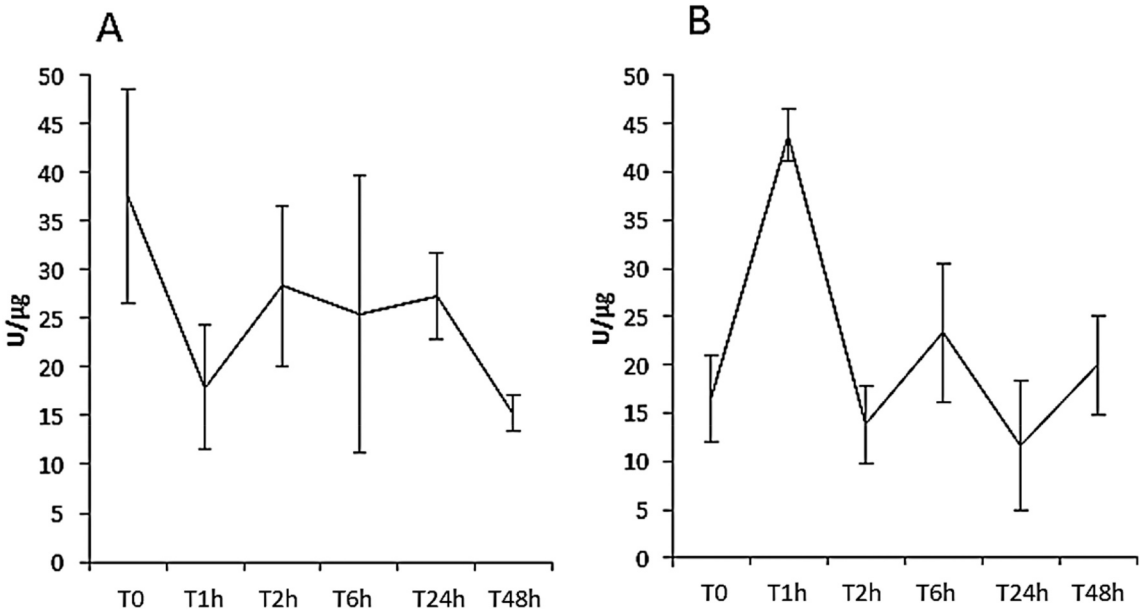

Fig. 3. A) Peroxidase activity in the CL samples from *H. tubulosa*; B) Peroxidase activity in the CfCf samples from the *H. tubulosa* specimens, measured at different post-wounding times (from T1h to T48h) and in the controls (T0). The values are expressed as the mean ± SD.

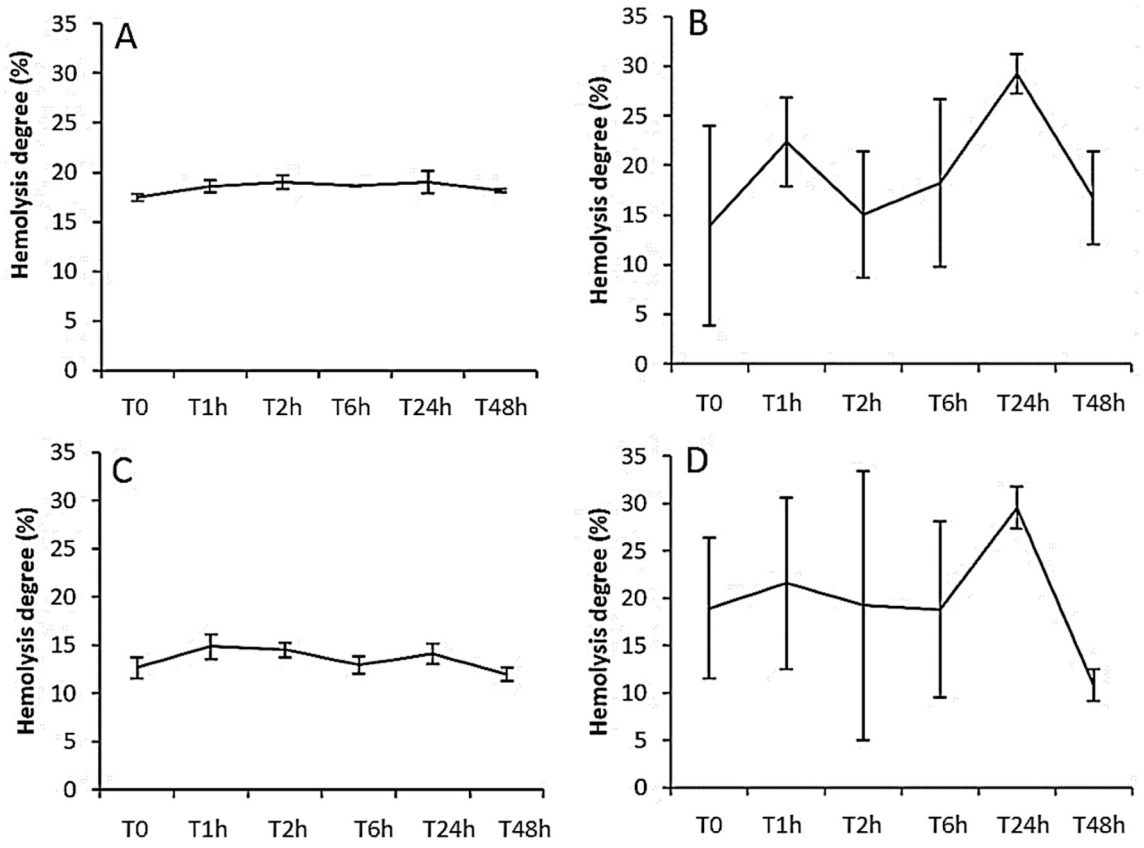

Fig. 4. Cytotoxic activity of *H. tubulosa*: A) CL, and B) CfCf tested against rabbit erythrocytes at different experimental times. Cytotoxic activity of *H. tubulosa*: C) CL, and D) CfCf tested against sheep erythrocytes at different times (from T1h to T48h) and in the controls (T0). The values are expressed as percentages of the total and as the mean ± SD.

an important role in the immunity of marine organisms (Mou et al., 1999; Liu et al., 2000; Ross et al., 2000; Sarlin and Philip, 2011), and they are commonly used as biomarkers in studies of environmental stress (Forget et al., 2003). They have also been described in the circulating coelomocytes of Holothuroids (Canicattì, 1990). However, although several papers have certainly increased what is known about these enzymes in this animal class (Liu et al., 2008; Wang et al., 2008; Kolasinski et al., 2010; Liu et al., 2012; Ma et al., 2013; Wu et al., 2013;

Wang et al., 2015; Wen et al., 2016; Li et al., 2018; Dang et al., 2019), no research to date has analyzed their activity during wound-healing processes. Our study is therefore the first to reveal that, at different times, there are significant increases in the activity of hydrolase enzymes in both cells and CfCf: in the former, the increases we identified were significant 1h after the cutting event; in the latter, the maximum activity occurred after 24h, signifying the enzymes' release into the circulation and involvement in wound-healing processes. This confirms that these enzymes: are more active within cells than CfCf (Jiang et al., 2017); and are released to a massive extent into the coelomic fluid under stressful conditions (Vazzana et al., 2020a). In fact, the elevated levels of enzymatic activity we identified inside the cells 1h after the cutting event could indicate: a high inflammatory state; a protein turnover that is typical of the regeneration process (Patruno et al., 2001); and a method of protection against invading pathogens (Ellis et al., 2011). It is known that the sea cucumber's immune response is non-specific (innate) (Wang et al., 2015); there is also awareness that (after phagocytic processes) these enzymes are the body's first line of internal defence and may help in the complete degradation of exogenous substances. Our findings therefore confirm that significant increases in enzymatic activity are induced by wound-stress conditions (Seitkalieva et al., 2016).

Previous studies have shown that stress affects the oxidative state of aquatic animals (Liu et al., 2015; Vazzana et al., 2020a, b). As in the case of hydrolase enzymes, the involvement in wound-healing of those from the peroxidase class has also been demonstrated (Kurahashi and Fujii, 2015). The roles of peroxidase in such processes are still poorly understood. It is, however, known that, while ROS are essential for protecting the body from infection, they can damage surrounding tissues and delay healing if they are present in excessive numbers (Kurahashi and Fujii, 2015). Moreover, there is awareness that, under stressful conditions, the levels of ROS like superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radicals (OH^-), and singlet oxygen ($^1O_2$) rise due to an imbalance between their production and removal (Fridovich, 1986; Asada and Takahashi, 1987). It is also known that highly reactive molecules can oxidize cellular components, causing oxidative stress (Halliwell and Gutteridge, 2001; Kong et al., 2012). Organisms are generally protected against the deleterious effects of ROS by a complex antioxidant system consisting of enzymatic and non-enzymatic detoxification mechanisms (Elstner, 1982; Smirnoff, 1993; Van Breusegem et al., 1998). Furthermore, antioxidant enzymes are the first line of defence against ROS in the sea cucumber, and include superoxide dismutase, catalase and peroxidase (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1999), which play a compensatory role in maintaining radical detoxification (Choi et al., 2000). Our study evaluated the activity of peroxidase in our CL and CfCf samples, because of its importance: as a biomarker of stress (Winston and Di Giulio, 1991); in wound-healing processes (Kurahashi and Fujii, 2015; Kümin et al., 2007); and in catalyzing the reduction of H_2O_2 (Kurahashi and Fujii, 2015). Our results suggest that this enzyme is released into the coelomic fluid before those belonging to the hydrolase class; these show an opposite trend, probably indicating that the two classes play different roles in wound healing. We also observed an opposite trend in the peroxidase activity 1h after cleavage, with a significant decrease in activity within the CL and a significant increase in the CfCf. These results concur with the findings of Hawa et al. (1999) and Vazzana et al. (2020a), and highlight that: the coelomic fluid in *H. tubulosa* has antioxidant properties; and the instantaneous release of the peroxidase enzyme following shear stress could be a strategy for addressing an immediate increase in the levels of ROS (Beyer et al., 1991; Ahmad, 1995; Vazzana et al., 2020a). Indeed, a lack of detoxification when there is an excessive production of ROS may cause lipid peroxidation (Halliwell and Gutteridge, 1999) and changes in the physical and chemical properties of cell membranes. This can impair cell functioning (Rikans and Hornbrook, 1997) and, as a consequence, wound healing (Kurahashi and Fujii, 2015). Our results confirm that, like other echinoderms, the activation of antioxidant defences in the coelomic fluid of *H. tubulosa* is a protective mechanism against increases in the levels of ROS (Fangyu et al., 2011). We also demonstrate for the first time the involvement of these defences in wound-response processes.

4.3. Cytotoxic activity of CL and CfCf

Cytotoxic activity, which is considered to be an efficient natural defence system in marine organisms (Cammarata et al., 2000), has been widely demonstrated and characterized in echinoderms (Bertheussen, 1979; Lin et al., 2001; Vazzana et al., 2020a). Echinoderms are exploited in medicine as a source of bioactive components, and are used for the extraction and purification of cytotoxic, haemolytic, anti-viral, anti-fungal, anti-microbial and anti-tumour components (Bordbar et al., 2011; Cardoso et al., 2016; Lee et al., 2014; Sharmin, 2017; Thao et al., 2014). This activity is mediated by the release of lysines in bodily fluids, is calcium-dependent (Canicatti, 1991; Pagliara and Canicattì, 1993; Arizza et al., 2007), and has been seen to produce a rapid and effective response, including towards tumour cells (Arizza et al., 2007). Wound healing is a complex procedure involving connective-tissue regeneration, and includes sequential and overlapping biological events entailing the proliferation and migration of different cell types (Sidhu et al., 1999). After injury, a wound must be repaired as quickly as possible to prevent microbial infection (Naik et al., 2009). In the Holothuroids, Canicattì et al. (1989) observed that the coelomocytes of *Holothuria polii* are able to lyse sheep erythrocytes (Canicattì, 1990; Canicattì et al., 1987). This was also seen in *H. tubulosa* in relation to rabbit and sheep erythrocytes (Vazzana et al., 2018). The current study demonstrated an increase in cytotoxic activity in CL samples in the first 2h after a wounding event. Also seen was a rise in this activity in CfCf samples after 24h, probably due to the activation of a production and protective release mechanism against pathogen entry, which was made possible because of the wound. In agreement with Vazzana et al. (2015), we also found that the degree of cytotoxicity returned to a similar level to that of the control specimens 48 h after the cutting event, which is consistent with the onset of wound healing. It is reported in the literature that the application of fish mucus can improve mammalian wound healing, because it is rich in bioactive molecules, including haemolytic components (Al-Hassan et al., 1983; Al-Hassan, 1990; Al-Hassan et al., 1991; Akunne et al., 2016). This could also be the case for the coelomic fluid of *H. tubulosa*.

5. Conclusions

For the first time, this study highlights two important factors relating to the immune response in echinoderms: (1) morphometric changes are involved in the wound-healing process; in particular, variations in length seem to be closely related to the changes we observed in the immune responses analyzed; and (2) typical humoral responses, and therefore enzymatic (i.e., hydrolase and antioxidant class-types) and cytotoxicity activity, are triggered at diverse times, highlighting their different roles in wound healing. We also provide evidence that the relative response at the cellular level is time-dependent and closely correlated with the response at the CfCf level. Finally, by exploiting one of the phylums with unique regenerative capacities, our work improves what is known about wound healing at the morphometric, physiological and immunological levels. Our findings point to the possibility of using the coelomic fluid of *H. tubulosa* in medicine and, therefore, for the treatment of wounds in human beings.

Funding

This work was supported by Fondo Finalizzato alla Ricerca di Ateneo (FFR, 2018-2021).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Vinicius Queiroz offers his personal thanks to FAPESP (Proc. 2015/21460-5 and 2018/14497-8). Thanks also go to Dr. Giampaolo Badalamenti for his collaboration in the management of the animals used in the experiments.

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