

RESEARCH ARTICLE

Open Access



Biochemical changes and drug residues in ascidian *Halocynthia roretzi* after formalin–hydrogen peroxide treatment regimen designed against soft tunic syndrome

Ji-Hoon Lee¹, Ju-Wan Kim¹, Yun-Kyung Shin², Kyung-Il Park¹ and Kwan Ha Park^{1*}

Abstract

Soft tunic syndrome (STS) is a protozoal disease caused by *Azumiobodo hoyamushi* in the edible ascidian *Halocynthia roretzi*. Previous studies have proven that combined formalin–hydrogen peroxide (H₂O₂) bath is effective in reducing STS progress and mortality. To secure target animal safety for field applications, toxicity of the treatment needs to be evaluated. Healthy ascidians were bathed for 1 week, 1 h a day at various bathing concentrations. Bathing with 5- and 10-fold optimum concentration caused 100% mortality of ascidians, whereas mortality by 0.5- to 2.0-fold solutions was not different from that of control. Of the oxidative damage parameters, MDA levels did not change after 0.5- and 1.0-fold bathing. However, free radical scavenging ability and reducing power were significantly decreased even with the lower-than-optimal 0.5-fold concentration. Glycogen content tended to increase with 1-fold bathing without statistical significance. All changes induced by the 2-fold bathing were completely or partially restored to control levels 48 h post-bathing. Free amino acid analysis revealed a concentration-dependent decline in aspartic acid and cysteine levels. In contrast, alanine and valine levels increased after the 2-fold bath treatment. These data indicate that the currently established effective disinfectant regimen against the parasitic pathogen is generally safe, and the biochemical changes observed are transient, lasting approximately 48 h at most. Low levels of formalin and H₂O₂ were detectable 1 h post-bathing; however, the compounds were completely undetectable after 48 h of bathing. Formalin–H₂O₂ bathing is effective against STS; however, reasonable care is required in the treatment to avoid unwanted toxicity. Drug residues do not present a concern for consumer safety.

Keywords: Ascidians, Formalin–hydrogen peroxide combination, Toxicity, Biochemical parameters, Drug residues, Soft tunic syndrome

Background

Soft tunic syndrome (STS) in the ascidian *Halocynthia roretzi* has markedly reduced production of this edible invertebrate. Official figures indicate a gradual decrease in production to less than a half of the peak yearly production of approximately 22,500 t in 1995 (Kumagai et al. 2010).

The cause of STS is infection with a protozoal parasite *Azumiobodo hoyamushi*, which leads to softening of the

rigid cellulose-protein tunic structure (Dache et al. 1992) without affecting the cellulose fiber structure itself (Kimura et al. 2015). Highly active protease enzymes are produced and excreted from *A. hoyamushi* cells (Jang et al. 2012). Although the disease spreads very rapidly, safe and effective measures have not been established to control the spread of STS in farms.

Chemical biocides are the first line of preventive measures against infective organisms in the absence of a practical method to deal with the infection. Different classes of biocidal agents have been tested, and formalin, H₂O₂, bronopol, povidone iodine, and NaOCl were found effective against the causative parasite (Park et al. 2014; Lee et al. 2016; Kumagai et al. 2016). The

* Correspondence: khpark@kunsan.ac.kr

¹Department of Aquatic Life Medicine, College of Ocean Science and Technology, Kunsan National University, 558 Daehak-ro, Miryong-Dong, Gunsan City, Jeonbuk 54150, Republic of Korea
Full list of author information is available at the end of the article



combination of two anti-infective chemicals, formalin and H₂O₂, was the most promising choice of treatment owing to their synergistic efficacy (Park et al. 2014). Detailed results from treatment trials were published in a previous issue of this journal (Lee et al. 2016), and this paper thus constitutes an important counterpart companion to it.

Bathing ascidians with formalin and H₂O₂ suggested a possible use for the combination in treating infected ascidians; however, the possible side effects, except mortality, have not been examined. Certain side effects are expected, considering the non-selective mechanisms of action of these agents. The degree and recovery from toxicity should be considered in deciding the value of a treatment regimen. To determine the toxicity and safety of the formalin–H₂O₂ treatment, overall mortality, biochemical changes, and drug residue levels were assessed after a 1-week bathing treatment schedule in healthy ascidians.

Methods

Chemicals

Formalin and H₂O₂ were purchased from Sigma (St. Louis, MO, USA), and actual concentrations were assessed before use by HPLC–UV (Soman et al. 2008) and peroxidase–H₂O₂ analysis kit (Cell Biolabs, San Diego, CA), respectively. All other reagents were purchased from Sigma if not specified otherwise.

Test animals: *Halocynthia roretzi*

Healthy ascidians (114.7 ± 21.9 g, 90.9 ± 15.5 cm long) were obtained from a local dealer and acclimated to laboratory conditions for 1 week before commencing the experiment. The absence of *A. hoyamushi* was verified by polymerase chain reaction (Shin et al. 2014) with 10 randomly sampled ascidians. Animals were maintained at 15 °C, the temperature at which STS is most likely to occur and treatment administration is expected. The aquaria used were rectangular PVC tanks (L 1.0 m × W 0.65 m × H 0.3 m water level, 195 L). During experiments, feeding was not performed and water exchange was not needed.

Treatment procedures

Drug treatment was performed at 10:00 a.m. for 1 h in separate drug tanks (20 L acryl baths) kept at 15 °C that were artificially aerated. After drug bathing, the ascidians were returned to normal tanks. This treatment was repeated once daily for a week. Control groups were kept in normal seawater. In toxicity tests, recovery was checked again 48 h after termination of bathing (48 h post-bath group) when tunic signs were detected in the initial assessment.

Assessment of oxidative damage and oxido-reductive potential

To estimate the influence of the treatment on oxido-reductive potential in treated animals, three different parameters were assessed: malondialdehyde (MDA) content, free radical scavenging activity, and reducing power of ascidian soft tissues.

MDA content was assessed by the thiobarbituric acid-reactive substance (TBARS) method (Ohakawa et al. 1979), using 10 g of tissues after homogenization in 20% trichloroacetic acid (TCA) solution. For this, the whole soft tissue of one individual was homogenized and 10 g was taken. 2-Thiobarbituric acid (5 mL, 5 mM; Sigma) was added to an equal volume of tissue homogenates and stored in a refrigerator before absorbance measurements at 530 nm (Optozen POP UV/Vis spectrophotometer, Meacasys, Seoul, Korea). Free radical scavenging activity was determined according to the DPPH scavenging method (Blios 1958). For this, 6 g of soft tissues were homogenized in 100 mL methanol followed by addition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) dissolved in methanol. The mixture was reacted for 10 min at room temperature, and absorbance was measured at 517 nm with a spectrophotometer.

Reducing power was determined (Oyaizu 1986) using the methanol-added homogenates described in the section describing the determination of free radical scavenging activity. Phosphate buffer (1 mL, 200 mM, pH 6.6) was added to 1 mL of tissue homogenate and mixed with 1% potassium ferricyanide solution (1 mL). After incubation at 50 °C for 20 min, 10% TCA was added to stop the reaction. Absorbance was measured at 700 nm using a spectrophotometer. Positive controls contained 10 μM ascorbic acid (vitamin C) instead of ascidian tissues.

Glycogen content

Glycogen content was analyzed according to the anthrone method (Roe and Dailey 1966). Minced soft tissues (2 g) were mixed with 30% KOH solution (1 mL) to hydrolyze glycogen to glucose. Final colored product obtained in reaction with anthrone was diluted with distilled water to appropriate concentrations before spectrophotometric measurements at 620 nm. The standard curve was prepared with D-glucose after identical processing.

Free amino acid composition

Free amino acids were analyzed according to the ninhydrin post-column derivatization method (Friedman 2004) optimized for the Hitachi amino acid analyzer (Hitachi L-8900, Hitachi, Tokyo, Japan). Soft tissues (5 g) were homogenized with distilled water (5 mL) and centrifuged at 3000 × g for 10 min at 3 °C. Next, to 1 mL of supernatant, 5% TCA (0.9 mL) was added to precipitate

proteins, followed by centrifugation at $5000 \times g$ for 10 min at 3°C . After 10-fold dilution of the supernatant with 0.02 N HCl, the samples were filtered through 0.2- μm membrane filters. The amino acids were separated with an ion exchange column (4.6×60 mm; Hitachi HPLC Packed Column No. 2622 Li type) installed in an amino acid analyzer and UV detector (Hitachi L-8900). The mobile phase was Wako buffer solution (L-8900 PF-1,2,3,4, Wako Pure Chemical Industries, Ltd., Osaka, Japan) run at a flow rate of 0.35 mL/min. Amino acid contents were quantified following a post-column ninhydrin reaction on-line with 0.3 mL/min ninhydrin solution flow. The separation column was kept at $30\text{--}70^\circ\text{C}$, and the ninhydrin reaction was carried out at 135°C . Individual amino acids were identified against the standard amino acid mixtures (Wako), with absorbance measured at 570 and 440 nm. The volume of the sample injection was 20 μL .

Analyses of formalin and H_2O_2

The bathing drug solutions and treated tissues were used for analyses of test drug concentrations. The bathing solution was analyzed directly after it was used for bathing without any further treatment. The ascidian tissues were homogenized in two volumes of distilled water and centrifuged to obtain supernatants. Formalin content was analyzed by HPLC–UV following complex formation with 2,4-dinitrophenylhydrazine (Soman et al. 2008). The limit of detection sensitivity was approximately 500 nM.

H_2O_2 analysis was performed using the OxiSelect hydrogen peroxide colorimetric assay kit (Cell Biolabs, San Diego, CA) in accordance with the manufacturer's instructions. The detection sensitivity limit was approximately 500 nM. Colored products were detected at 540 nm and quantified by comparison with the standard curve.

Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Statistical analyses performed on biochemical parameters were conducted by one-way analysis of variance followed by Duncan's multiple comparison tests. Significance in the difference of means was declared for p values <0.05 .

Results

Mortality of ascidians

Figure 1 illustrates the mortality of ascidians following bathing treatment with formalin– H_2O_2 combination. Ascidians were treated for 1 h a day over a week at indicated concentrations, and survival was recorded. Since the optimum anti-parasitic treatment under identical conditions was formalin: $\text{H}_2\text{O}_2 = 40:10$ ppm (determined

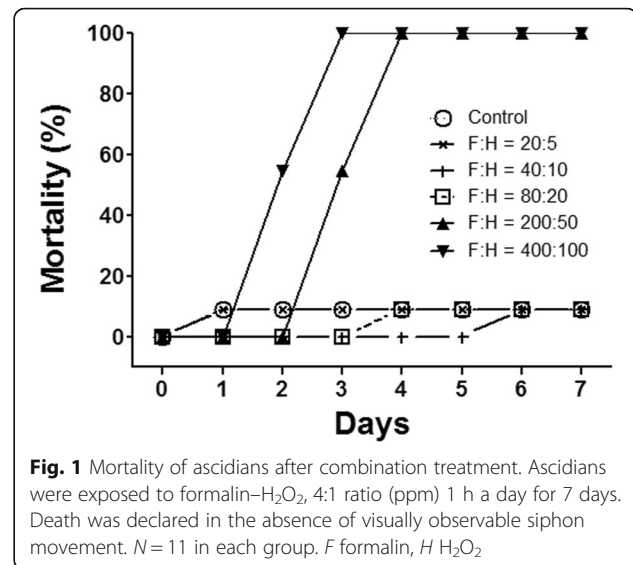


Fig. 1 Mortality of ascidians after combination treatment. Ascidians were exposed to formalin– H_2O_2 , 4:1 ratio (ppm) 1 h a day for 7 days. Death was declared in the absence of visually observable siphon movement. $N = 11$ in each group. F formalin, H H_2O_2

in previous studies, see refs. (Park et al. 2014; Lee et al. 2016)), the treatments corresponded to exposure from 0.5- (20:5 ppm) to 10-fold (400:100 ppm) optimal treatment. The mortality was concentration-dependent.

Whereas 0.5- to 2.0-fold treatments caused 10% mortality, not different from that by the non-treated control, 5- and 10-fold bathing led to 100% ascidian mortality.

Oxidative damage and oxido-reductive potential

Oxidative damage and the effect of treatment on the oxido-reductive potential of ascidian soft tissues are shown in Fig. 2. These parameters were determined 24 h post-bathing after the termination of the 1 week exposure schedule. Bathing of ascidians with 2-fold optimum concentration caused a slight but significant elevation of lipid peroxide levels after 1 week exposure for 1 h a day (Fig. 2a). In addition, free radical scavenging activity was diminished by exposure to treatment: significant reduction was noticed even after 0.5-fold exposure (Fig. 2b). Along with the reduction in free radical scavenging, a significant decrease in reducing power was observed in the same tissues (Fig. 2c). Addition of vitamin C to control tissues markedly elevated the reduction potential, as indicated by elevated free radical scavenging capacity and reducing power (Figs. 2b, c). These alterations returned to pre-treatment levels after 48-h recovery in fresh seawater.

Tissue glycogen content

A biphasic pattern in glycogen levels was observed (Fig. 3). The levels increased in a concentration-dependent manner after 0.5- and 1-fold treatments and returned to control levels after 2-fold exposure. Glycogen content stayed unchanged 48 h after exposure when kept in fresh seawater.

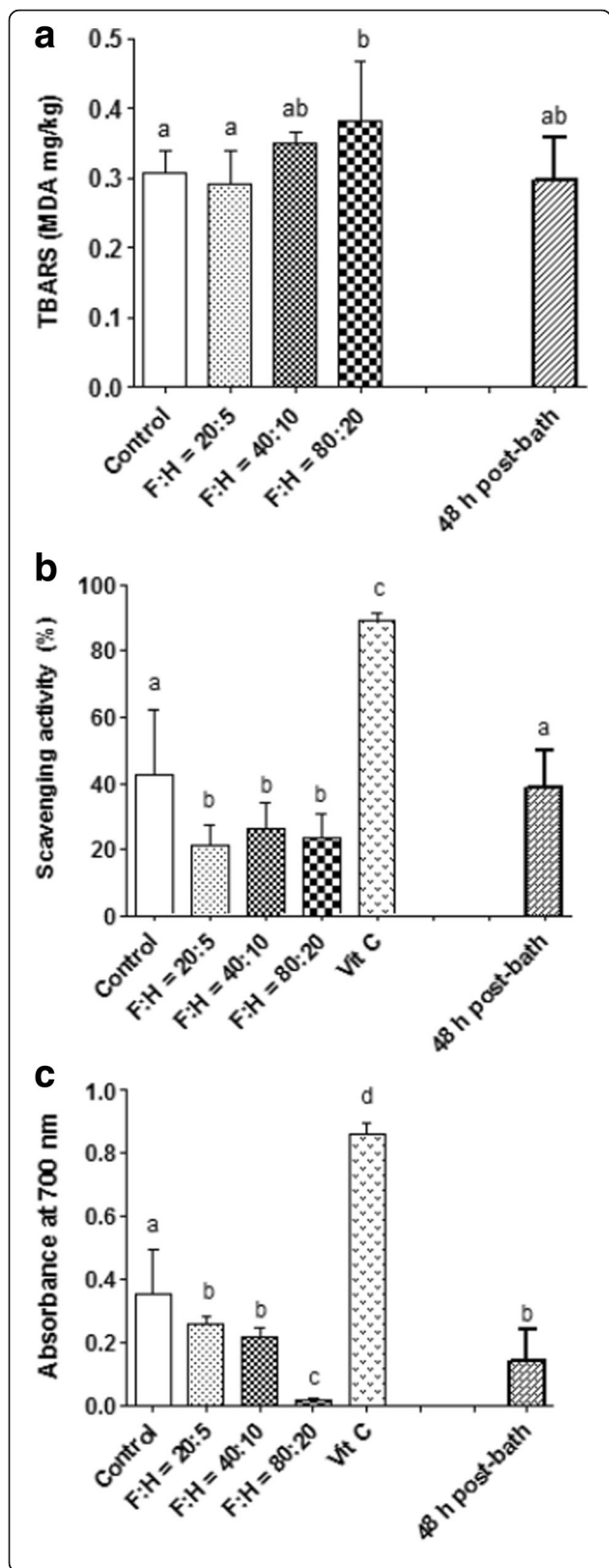


Fig. 2 Levels of oxidative damage in soft tissues of ascidians exposed to the combination treatment. Each damage parameter was assessed 24 h after the termination of the whole 7-day exposure scheme. Recovery (48 h post-bath column) was assessed in 2-fold exposure (F:H = 80:20 ppm) group after an additional 24 h in fresh seawater. **a** Malondialdehyde levels. **b** Free radical scavenging capacity. **c** Reduction power. F formalin, H H₂O₂, Vit C ascorbic acid (10 μM). N = 7. Superscripts over bars denote significant statistical difference by Duncan’s multiple comparison tests at *p* < 0.05

Free amino acid composition

Free amino acid composition of the edible tissues following drug bathing is shown in Table 1. Taurine was the most prevalent amino acid-like substance, followed by amino acids proline, glutamic acid, glycine, and histidine. The most evident treatment-induced change was a concentration-dependent, significant decrease in aspartic acid concentration. In addition, significant decreases were noted for cysteine levels after 2-fold treatment and proline levels after 0.5-fold exposure. Notably, a significant increase in alanine and valine content was observed after 2-fold exposure.

Formalin and H₂O₂ residue concentrations

Drug residue concentrations in the bathing solution and treated ascidian tissues are shown in Fig. 4. Optimal, 1-fold treatment was used for the residue analysis experiment (40 ppm formalin and 10 ppm H₂O₂). Formalin concentration in the bathing solution was approximately 30 ppm after use for 1 h and declined slowly over the

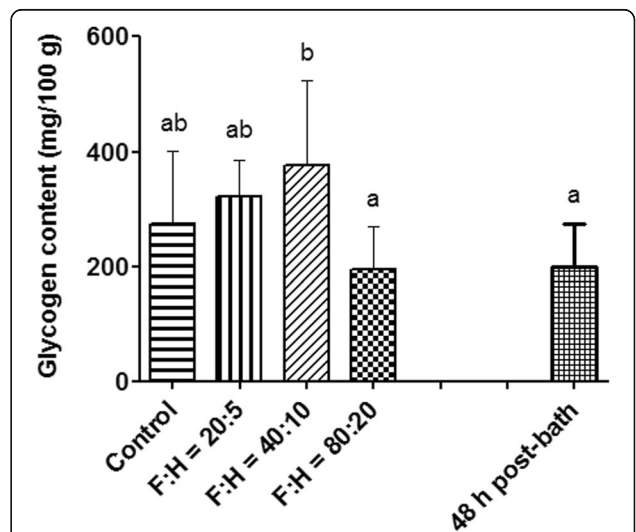


Fig. 3 Glycogen contents of soft tissues in ascidians exposed to combination treatment. Glycogen content was assessed 24 h after the termination of the whole 7-day exposure scheme. Recovery (48 h post-bath column) was assessed in 2-fold exposure (F:H = 80:20 ppm) group after an additional 24 h in fresh seawater. F formalin, H H₂O₂. N = 7. Superscripts over bars denote significant statistical difference by Duncan’s multiple comparison tests at *p* < 0.05

Table 1 Free amino acid content of edible tissues exposed to the treatment

Amino acids	Control (n = 7)	Amino acid content (mg/100 g muscle)		
		0.5 × (n = 10)	1 × (n = 10)	2 × (n = 9)
Taurine (Tau)	1860.36 ± 357.57	1526.89 ± 186.85	1816.14 ± 242.77	1601.10 ± 247.87
Aspartic acid (Asp)	98.34 ± 47.38 ^a	75.68 ± 30.71 ^{ab}	57.42 ± 16.54 ^{bc}	37.61 ± 28.71 ^c
Threonine (Thr)	72.56 ± 29.51	78.14 ± 24.18	70.97 ± 22.51	87.15 ± 31.46
Serine (Ser)	47.49 ± 13.97	47.24 ± 10.11	57.21 ± 14.36	61.54 ± 20.62
Glutamic acid (Glu)	305.64 ± 54.55	248.89 ± 67.42	309.87 ± 67.07	295.48 ± 70.03
Glycine (Gly)	238.45 ± 57.63	183.81 ± 30.83	207.38 ± 33.59	183.10 ± 48.06
Alanine (Ala)	97.86 ± 22.88 ^a	89.12 ± 13.86 ^a	98.40 ± 18.56 ^a	142.61 ± 45.83 ^b
Citrulline (Cit)	0.29 ± 0.76	0.83 ± 2.61	2.26 ± 3.04	0.90 ± 1.45
Valine (Val)	23.85 ± 7.56 ^a	27.13 ± 10.06 ^{ab}	22.67 ± 10.05 ^a	36.07 ± 13.19 ^b
Cysteine (Cys)	5.22 ± 3.00 ^a	3.80 ± 3.80 ^{ab}	5.38 ± 2.88 ^a	1.45 ± 1.66 ^b
Methionine (Met)	15.09 ± 6.93	12.47 ± 5.04	11.71 ± 4.42	12.10 ± 3.42
Isoleucine (Ile)	19.08 ± 6.26	20.28 ± 7.14	17.67 ± 6.44	25.44 ± 8.85
Leucine (Leu)	27.92 ± 7.88	34.92 ± 14.07	26.16 ± 9.72	39.49 ± 11.85
Tyrosine (Tyr)	44.50 ± 21.21	27.68 ± 13.18	35.48 ± 28.24	46.99 ± 20.80
Phenylalanine (Phe)	23.20 ± 5.38	32.51 ± 8.87	25.86 ± 6.54	29.99 ± 7.62
Ornithine (Orn)	2.61 ± 0.80	2.49 ± 0.75	2.58 ± 0.74	2.31 ± 0.70
Lysine (Lys)	34.67 ± 8.98	38.28 ± 12.12	36.35 ± 6.27	33.25 ± 5.63
Histidine (His)	144.42 ± 40.33	120.30 ± 37.19	151.46 ± 49.64	145.57 ± 29.00
Arginine (Arg)	15.26 ± 4.53	19.46 ± 7.67	13.99 ± 4.21	12.66 ± 4.80
Proline (Pro)	688.83 ± 286.60 ^a	453.67 ± 139.60 ^b	607.11 ± 116.11 ^{ab}	558.56 ± 96.81 ^{ab}

Values with different superscript letters are significantly different ($p < 0.05$)

next 24 h (Fig. 4a). The tissue formalin concentrations in the ascidians were approximately 1/3 of the bath concentration after 1 h and undetectable after 48 h (Fig. 4b). H₂O₂ concentrations exhibited a similar pattern to formalin; the agent was stable in the seawater bath and barely detectable in ascidian tissues after 24 h (Fig. 4c, d). The lowest concentrations of formalin and H₂O₂ were about 0.4 and 0.1 ppm, respectively. These concentrations apply for both ascidian tissues and culture water.

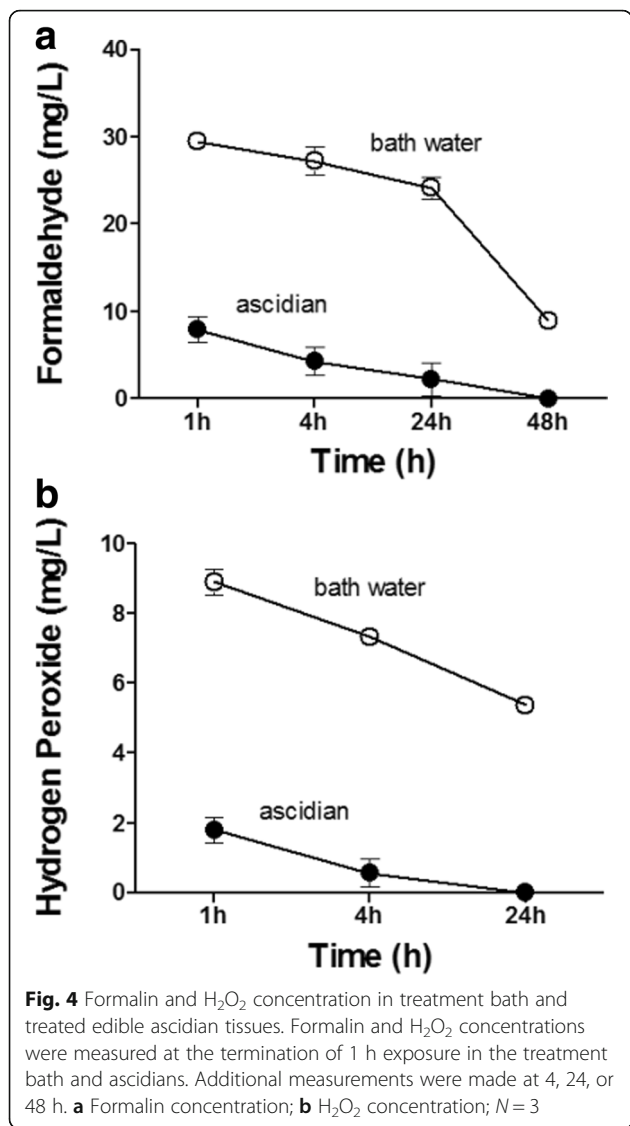
Discussion

This study was performed to assess the toxicity of combined formalin–H₂O₂ treatment in edible ascidians. Formalin–H₂O₂ combination is very effective against the tunic-infecting parasite *A. hoyamushi* (Park et al. 2014; Lee et al. 2016). The treatment concentrations tested here were based on concentrations exerting anti-protozoal effects and used for treating STS. Biochemical responses were monitored to evaluate the toxic effects of the formalin–H₂O₂ combination. Drug residue concentrations were analyzed to correlate toxicity with drug levels in the tissue.

Oxidative damaging effects of the combined agents were evaluated by examining lipid peroxidation, free radical scavenging activity, and reduction potential in edible

tissues. Significant changes in these parameters were observed at optimal treatment concentrations of 40 ppm formalin and 10 ppm H₂O₂. H₂O₂ exposure stimulates lipid peroxidation, as H₂O₂ biocidal effects in living organisms are based on production of free radicals (Siddique et al. 2012; Cavaletto et al. 2002). In addition, formaldehyde causes lipid peroxidation (Gulec et al. 2006; Saito et al. 2005) directly and via a secondary mechanism involving the production of reactive oxygen species (Hancock et al. 2001). Although further studies are required, it is reasonable to assume that the combined formalin–H₂O₂ treatment stimulated lipid peroxidation at the 2-fold effective concentration in this marine invertebrate. It is known that reactive oxygen species deplete endogenous reducing biomaterials in cells (Lushchak 2014) and glutathione is the representative reducing agent in marine invertebrates (Conners 1998). Lipid peroxidation is postponed until reducing reserves of the cell are completely exhausted. The observed pronounced decline in free radical scavenging ability and reducing power compared to elevation of lipid peroxidation could indicate that some biochemical changes occur than others.

Major glycogen deposits in ascidians occur in the pyloric gland, which plays a homologous role in the liver



in other animals (Ermak 1977). Glycogen storage in the pyloric gland indicates disturbed metabolic activity (Gaill 1980), and thus, the increasing trend after 1-fold exposure reflects a perturbation in energy balance caused by the treatment. However, interpretation becomes complicated, as the 2-fold exposure did not increase glycogen content. It is known that reduction status induce changes in glycogen contents in mice (Nocito et al. 2015).

Free amino acid content is an indicator of toxic response in various aquatic invertebrate animals (Cook et al. 1972; Hosoi et al. 2003). Changes in free amino acid levels in tissues during stress occur because of altered amino acid utilization for protein synthesis (Kültz 2005). Amino acid changes in ascidians occur seasonally (Watanabe et al. 1983). However, changes in amino acid composition in response to chemical stress have not been studied in *H. roretzi*.

Observed amino acid patterns match the typical characteristics of edible tissues of this species: high content of taurine, proline, glutamic acid, and glycine, as described by Watanabe (Watanabe et al. 1983). Aspartic acid concentration was reduced in a concentration-dependent manner. Although aspartic acid levels were relatively high (taurine > proline > glutamic acid, glycine, histidine > aspartic acid, alanine, threonine>serine), the importance of this amino acid in physiology of ascidians is not known. Cysteine content was diminished after 2-fold exposure. Reduced cysteine content may reflect the changes in reducing potential because cysteine is used as a precursor in the synthesis of glutathione, which reactive compounds like formalin and H₂O₂ may deplete (Poole 2015). Cysteine protects against free radical damage caused by paraquat (Shoji et al. 1992), although the significance of cysteine in ascidians is not known.

A very interesting phenomenon observed in the free amino acid analysis was the elevation of alanine and valine levels. Alanine is important for intracellular osmolality regulation in Pacific oysters, with salinity changes inducing immediate elevation of alanine levels in mantle tissues (Hosoi et al. 2003). However, the importance of alanine and valine in ascidians in relation to stress requires further studies.

Biochemical toxic responses were observed 24 h post-bathing; however, associated residue levels of formalin and H₂O₂ were undetectably low. This finding implies that the exposed animals were recovering and further progression of toxicity is not expected. In addition, rapid elimination of treatment agents is ideal from the aspect of food safety. In contrast to the rapid decline of formalin and H₂O₂ residues in edible ascidian tissues, the compounds are reasonably stable in aquatic media, making daily 1 h bathing treatment possible (Jung et al. 2001; Yamamoto et al. 2011). In addition to their role in the main purpose of this study, which is examining toxic responses to formalin–H₂O₂ bath treatments, biochemical parameters assessed can be used to monitor the impact of these chemicals during treatment.

In view of toxicity from this study, formalin–H₂O₂ bathing sounds promising to disinfect ascidians against STS-causing parasites. The practice will be specifically useful before landing ascidian seedlings in Korean waters.

Conclusions

STS is a highly infectious protozoal disease that has severely affected ascidian industry in Asian countries. Bathing treatment with formalin–H₂O₂ combination solution is an effective method for reducing STS mortality. Bathing treatment with optimal drug concentrations induces a certain recoverable level of biochemical changes. Our results indicate that the two biocidal drugs studied possess inherent toxicity related to their mechanisms of

action. However, the treatment toxicity is acceptable as long as excess bathing concentrations are avoided. After treatment, both drugs are eliminated relatively quickly from edible ascidian tissues.

Abbreviations

STS: Soft tunic syndrome; TCA: Trichloroacetic acid; MDA: Malondialdehyde

Acknowledgements

We thank the Marine Biology Education Center of the Kunsan National University for providing the ascidian culture facilities during the experiment.

Funding

This work was financially supported by the National Fisheries Research and Development Institute of Korea (NFRDI, RP-2016-AQ-038).

Availability of data and materials

Not applicable

Authors' contributions

JHL carried out the bathing treatments, sample preparations, and necessary chemical and biochemical analyses. KIP performed the tissue histology on the treated ascidian specimens. YKS, KIP, and KHP participated in the writing of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval

No approval is needed for invertebrate animal studies from the Institutional Animal Care and Use Committee of the Kunsan National University.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Department of Aquatic Life Medicine, College of Ocean Science and Technology, Kunsan National University, 558 Daehak-ro, Miryong-Dong, Gunsan City, Jeonbuk 54150, Republic of Korea. ²Southeast Sea Fisheries Institute, National Institute of Fisheries Science, Younggwun-Ri 361, Sanyang-Eup, Tongyeong City, Kyungnam, Republic of Korea.

Received: 13 March 2017 Accepted: 29 June 2017

Published online: 11 July 2017

References

- Blios MS. Antioxidant determination by the use of a stable free radical. *Nature*. 1958;181:1199–200.
- Cavaletto N, Ghezzi A, Burlando B, Evangelisti V, Cerrato N, Viarengo A. Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis*. *Comp Biochem Physiol Part C*. 2002;131:447–55.
- Conners DE. Master's Thesis. Medial University of South Carolina/University of Charleston-South Carolina, Environmental Studies Program. Charleston, SC, USA. 1998
- Cook PA, Gabbott PA, Youngson A. Seasonal changes in the free amino acid composition of the adult barnacle, *Balanus balanoides*. *Comp Biochem Physiol*. 1972;42B:409–21.
- Dache YB, Revol JF, Gail F, Goffinet G. Characterization and supramolecular architecture of the cellulose-protein fibrils in the tunic of the sea peach (*Halocynthia papillosa*, Ascidiacea, Urochordata). *Biol Cell*. 1992;76:87–96.
- Ermak TH. Glycogen deposits in the pyloric gland of the ascidian *Styela clava* (Urochordata). *Cell Tissue Res*. 1977;176:47–55.
- Friedman M. Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *J Agric Food Chem*. 2004;52:385–406.
- Gaill F. Glycogen and degeneration in the pyloric gland of *Dendrodos grossularia* (Ascidiacea, Tunicata). *Cell Tissue Res*. 1980;208:197–206.
- Gulec M, Gurel A, Armutcu F. Vitamin E protects against oxidative damage caused by formaldehyde in the liver and plasma of rats. *Braz J Med Biol Res*. 2006;34:639–43.
- Hancock JT, Desikan R, Neill SJ. Role of reactive oxygen species in cell signaling pathways. *Biochem Soc Trans*. 2001;29:343–50.
- Hosoi M, Kubota S, Toyohara M, Toyohara H, Hayashi I. Effect of salinity change on free amino acid content in Pacific oyster. *Fish Sci*. 2003;69:395–400.
- Jang HB, Kim YK, del Castillo CS, Nho SW, Cha IS, Park SB, Ha MA, Hikima JI, Hong SJ, Aoki T, Jung TS. RNA-seq-based metatranscriptomic and microscopic investigation reveals novel metalloproteases of *Neobodo* sp. as potential virulence factors for soft tunic syndrome in *Halocynthia roretzi*. *PLoS One*. 2012;7:e52379.
- Jung SH, Kim JW, Jeon IG, Lee YH. Formaldehyde residues in formalin-treated olive flounder (*Paralichthys schlegelii*), and seawater. *Aquaculture*. 2001;194:253–62.
- Kimura S, Nakayama K, Wada M, Kim EJ, Azumi K, Ojima T, Nozawa A, Kitamura SI, Hirose E. Cellulose is not degraded in the tunic of the edible ascidian *Halocynthia roretzi* contracting soft tunic syndrome. *Dis Aquat Org*. 2015;116:143–8.
- Kültz D. Molecular and evolutionary basis of the cellular stress response. *Ann Rev Physiol*. 2005;67:225–7.
- Kumagai A, Suto A, Ito H, Tanabe T, Takahashi K, Kamaishi T, Miwa S. Mass mortality of cultured ascidians *Halocynthia roretzi* associated with softening of the tunic and flagellate-like cells. *Dis Aquat Org*. 2010;90:223–34.
- Kumagai A, Tanabe T, Nawata A, Suto A. Disinfection of fertilized eggs of the edible ascidian *Halocynthia roretzi* for prevention of soft tunic syndrome. *Dis Aquat Org*. 2016;118:153–8.
- Lee JH, Lee JG, Zeon SR, Park KI, Park KH. Methods to eradicate soft tunic syndrome (STS)-causing protozoa *Azumiobodo hoyamushi*, the highly infectious parasite from the edible ascidian (*Halocynthia roretzi*). *Fish Aquat Sci*. 2016;19:1–6.
- Lushchak VI. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chem Biol Inter*. 2014;224:164–75.
- Nocito L, Kleckner AS, Yoo EY, Jones AR, Liesa M, Corkey BE. The extracellular redox state modulates mitochondrial function, gluconeogenesis, and glycogen synthesis in murine hepatocytes. *PLoS One*. 2015;10:1–17.
- Ohakawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95:351–8.
- Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jpn J Nutr*. 1986;7:307–15.
- Park KH, Zeon SR, Lee JG, Choi SH, Shin YK, Park KI. *In vitro* and *in vivo* efficacy of drugs against the protozoan parasite *Azumiobodo hoyamushi* that causes soft tunic syndrome in the edible ascidian *Halocynthia roretzi* (Drasche). *J Fish Dis*. 2014;37:309–17.
- Poole BS. The basics of thiols and cysteines in redox biology and chemistry. *Free Rad Biol Med*. 2015;80:148–57.
- Roe JH, Dailey RE. Determination of glycogen with the anthrone reagent. *Anal Biochem*. 1966;15:245–50.
- Saito Y, Nishio K, Yoshida Y, Niki E. Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. *Toxicology*. 2005;210:235–45.
- Shin YK, Nam KW, Park KH, Yoon JM, Park KI. Quantitative assessment of *Azumiobodo hoyamushi* distribution in the tunic of soft tunic syndrome-affected ascidian *Halocynthia roretzi* using real-time polymerase chain reaction. *Parasites Vectors*. 2014;7:539–43.
- Shoji K, Yuri M, Toshiya H, Moriyko K, Hideaki S, Tayayuki F. Effect of C-cystine on toxicity of paraquat in mice. *Toxicol Lett*. 1992;60:75–82.
- Siddique YH, Ara G, Afzal M. Estimation of lipid peroxidation induced by hydrogen peroxide in cultured human lymphocytes. *Dose-Response*. 2012;10:1–10.
- Soman A, Qiu Y, Li QC. HPLC-UV method development and validation for the determination of low level formaldehyde in a drug substance. *J Chromatogr Sci*. 2008;46:461–5.
- Watanabe K, Maezawa H, Nakamura H, Konosu S. Seasonal variation of extractive nitrogen and free amino acids in the muscle of the ascidian *Halocynthia roretzi*. *Bull Jpn Soc Sci Fish*. 1983;49:1755–8.
- Yamamoto A, Toyomura S, Saneyoshi M, Hatai K. Control of fungal infection of salmonids by hydrogen peroxide. *Fish Pathol*. 2011;36:241–6 (in Japanese).