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Developmental Abnormalities in Zebrafish Angiogenesis with Chronic Exposure to Crude Oil and Dispersant

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Abstract - Oil spills have occurred throughout the years of industrialization and represent a global challenge as they affect vast areas of the ocean. The toxicity of crude oil to aquatic organisms has been extensively investigated, but the potential impacts of crude oil on vertebrate development remain largely unknown. Here, we investigated the effects of dispersants used in treating a recent oil spill, as well as that of crude oil, on vertebrates by using the zebrafish (*Danio rerio*) model species, which has been widely used in empirical studies of both early embryonic development and adult physiology. Chronic exposure to crude oil resulted in marked developmental abnormalities, including pericardial edema, abnormal trunk vessel development, retardation of axonal branching, and abnormal jaw development. Embryonic development was affected more severely by exposure to the oil-dispersant combination than to the oil alone. Thus, the zebrafish *in vivo* model system suggests that dispersant treatment can have detrimental developmental effects on vertebrates and its potential impact on marine life, as well as humans, should be carefully considered in clean-up efforts at the site of an oil spill.

Key words : zebrafish, crude oil, dispersant, Olig2, Flk1

INTRODUCTION

As many countries have become industrialized, the need for oil has increased. The worldwide exportation has been accompanied by an overall increase in environmental pollution directly related to transport of this commodity. More and more cases of oil spills have occurred, and these spills are an enormous problem as they affect vast areas of the ocean. Scientists now believe that shrimp, fish, and crabs in the spill area have also experienced abnormal development as a result of the chemicals that are subsequently released to disperse the spilt oil (Schrope 2011). Although lighter fractions of crude oil are highly toxic and can cause cancer, they also evaporate quickly and cause less harm in oil spills (Mehlman and Legator 1991). However, the heavier fractions of crude oil are more persistent because they stay in the water for longer periods and have more affect on the sea life (Fleeger *et al*. 2003). A major effect of spilled oil is its coverage of the water's surface, which prevents light and oxygen from penetrating into the water. This effect becomes a problem for plankton and other plants whose main source of nutrition is photosynthesis. Seabirds and other animals are also affected by the oil spill as they become covered with oil. Oil coating animal fur or feathers reduces their insulating effects. In the case of seabirds, the oil coating their plumage can prevent flight and stress the bird's immune system (Briggs *et al*. 1996).

After an oil spill takes place, the immediate course of ac-

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tion is to float an oil fence, which prevents further spread of the oil. In addition, skimmers are used to remove the oil floating on the surface of the water (Broje and Keller 2006). There are three kinds of skimmers: weir, oleophilic, and drum. Weir skimmers work by letting the oil floating on the surface of the water flow over the weir (Robert 1998), which continues to let water flow into it until the oil is gone from the surface. Oleophilic skimmers, in contrast, function by rotating a drum that the oil adheres to. The oil is then physically wiped from the drum and collected. Drum skimmers do not pick up appreciable amounts of water, even when oil is not present (Robert 1998). Sorbents are also used to remove the oil and were widely used in treating the 2007 Korean oil spill.

If the layer of oil is too thin, it is difficult to remove physically. Therefore, chemical methods are necessitated. Dispersants are the most widely used chemical method. Dispersants are biochemically stable, non-ionic, surface-active agents, and their properties rely on the size of the hydrophilic and hydrophobic groups present in each molecule (Chapman *et al*. 2007). The dispersant acts to disperse liquids which do not generally mix with water. Surface-active agents are amphipathic compounds which possess both a hydrophilic group and a lipophilic group in one molecule. With water and oil alone, one cannot create a stable emulsion. The dispersant helps to scatter the oil equally throughout the water.

In 2007, a barge collided with an anchored ship carrying 260000 tons of crude oil, causing an oil spill of ~12000 tons into the sea near Taean County in the Chung Nam Province of South Korea. As part of the restoration process, the government had floated oil fences in the sea to minimize the spread of oil (Wolf 2008). Furthermore, by using about 268710 kilograms of oil sorbents and other clean-up devices, ~4000 tons of crude oil were collected (Wolf 2008). Additional research conducted by *Citizens Institute for Environmental Studies, Daejeon* revealed that the amount of benzene was very high in all the beaches and nearby residential areas at the oil spill site (Kim 2007). Benzene is a potent carcinogen, as designated by the International Agency for Research on Cancer, World Health Organization, Environmental Protection Agency, and other worldwide organizations. Out of 24 sites surveyed, 19 (79%) had benzene levels over the 2010 Korean standard limit (1.5 ppb) and all surpassed the Japanese standard limit (0.94 ppb) (Kim 2007). Other harmful carcinogenic substances and heavy metals, such as toluene, mercury and xylene, were found in very high amounts in the atmosphere of the beaches at the oil spill site. In addition, some of these substances were found in the blood and urine of the clean-up volunteers and local residents, causing substantial anxiety regarding the effects of this exposure (Kim 2007). Dispersant usage was one of the main methods of treating this oil spill. However, the dispersants might have created larger problems in the environment than the crude oil floating along the coast. Therefore, in this study, we examined the effects of the dispersants used in the oil spill, as well as that of crude oil alone on vertebrate development and physiology by using the zebrafish (*Danio rerio*) as an *in vivo* model system, which has been widely used in studies of both early embryonic development and adult physiology.

MATERIALS AND METHODS

1. Fish maintenance and strains

Zebrafish were maintained as previously described (Westfield 1995). The fluorescent reporter lines were *Tg[olig2:DsRed]* (Son *et al*. 2009), *Tg[flk1-GFP]* (Jin *et al*, 2005), and *Tg[her4:EGFP]* (Yeo *et al*. 2007). To analyze developmental abnormalities of the nervous system, we used a transgenic zebrafish line in which DsRed expression is controlled by the zebrafish *olig2* promoter (Son *et al*. 2009). Motor neuron and oligodendrocyte precursors express *olig* genes, which encode basic helix-loop-helix transcription factors that play important roles in the development of both cell types (Son *et al*. 2009). To facilitate analysis of angiogenesis, we used *Tg[flk1:GFP]* transgenic zebrafish line in which GFP expression is controlled by the zebrafish *flk-1* promoter (Jin *et al*. 2005). Flk-1 is one of the receptor tyrosine kinases comprising the vascular endothelial growth factor (VEGF) receptor family (Jin *et al*. 2005). When the basic vascular system of the whole embryo has been established and circulation can be observed at 26 hpf, the transcripts of *flk-1* were clearly detected in all the endothelial cells lining the vasculature (Jin *et al*. 2005). Thus, *Tg[flk1:GFP]* transgenic zebrafish, which permit visualization of endothelial cells for *in vivo* study of toxicants that disrupt cardiovascular development.

2. Oil and dispersant treatments

Adult zebrafish and embryos were treated with either oil, dispersant, or both, and the activity and survival of the animals were monitored. Glass bottles (~600 mL capacity) were filled with 500 mL of water and used to create water accumulated fractions (WAF) of crude oil according to conventional methods (Singer *et al*. 2000). The treatments were as follows: control, water alone; oil (2 g); dispersant (0.6 mL); oil (2 g) plus dispersant (0.6 mL). The oil used was collected from Mallipo Beach, Taean, where the 2007 oil spill took place, and the density of the crude oil was ~0.8 g mL^{-1}. The (v : v) ratio for water:oil was 200 : 1 (v : v) and oil : dispersant was 12.5 : 3, both of which were designed to approximate the condition of the oil spill. The dispersant used was SG-1000 (Kwang Woo Parker Co., Ltd., Korea), an O/W type dispersant, which was the dispersant used to treat the 2007 Korean oil spill. Two female and three male zebrafish were placed in each bottle and the movements of each zebrafish were recorded in 10 min intervals using a camcorder. Fifty zebrafish embryos were exposed to each of the various treatments starting at 12 hours post-fertilization (hpf) until a maximum of three days post-fertilization (dpf).

3. *In vivo* fluorescence imaging

The transgenic zebrafish were imaged at various times using the following protocol. To facilitate better observation, the chorion of each embryo was removed at 28 hpf, 48 hpf, or 72 hpf using sharp forceps to gently tear the chorion so that the embryo would fall out uninjured when the chorion was turned upside down. At the end of each exposure time course, the zebrafish were anesthetized in 0.1 mg mL^{-1} tricaine (Sigma, St. Louis, MO, USA), placed in a 60 mm diameter petridish filled with 4 mL of 1% agarose, and embedded in low-melting 0.7% agarose (FMC,) containing 0.1 mg mL^{-1} tricaine. After the agarose solidified, the zebrafish were imaged using a MVX10 fluorescence microscope (Olympus,

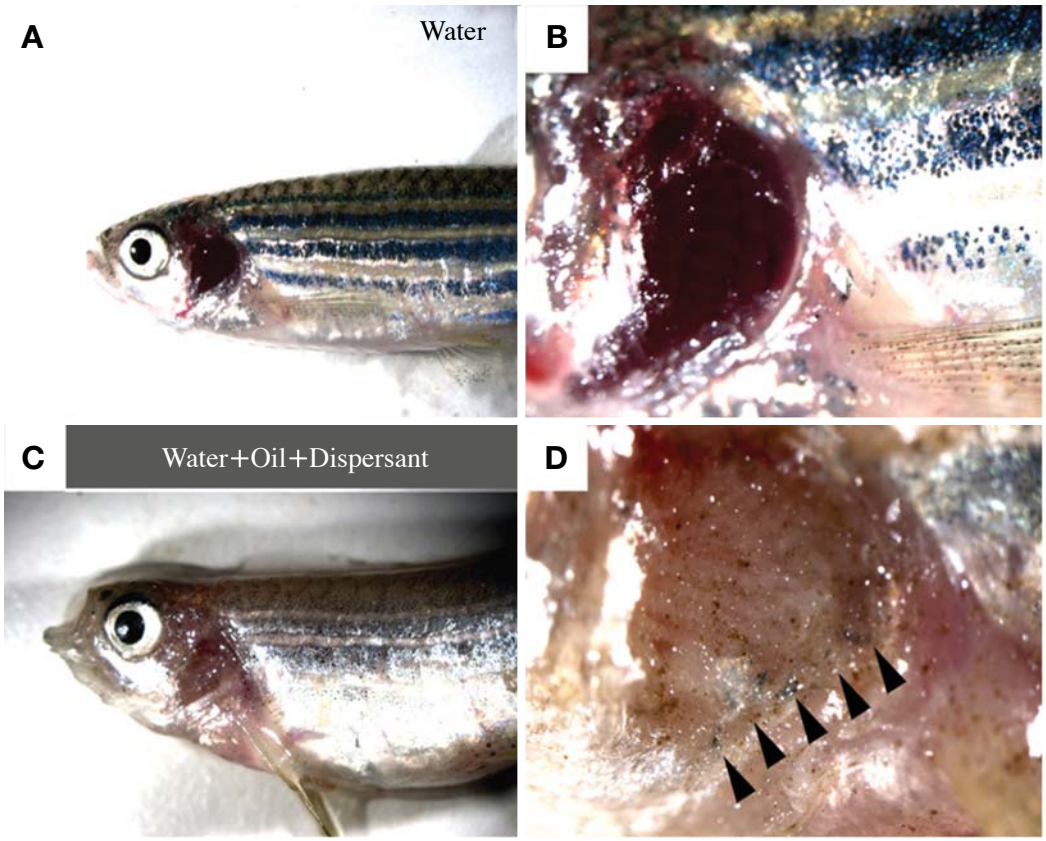

Fig. 1. Effects of oil and dispersant on adult zebrafish. Adult zebrafish incubated in water (A). Crude oil was detected in the gills of 6-month-old zebrafish exposed to the oil/dispersant combination (C). High magnification view of gills in control fish (B) and in oil/dispersant-treated fish (D).

Tokyo, Japan). To determine the growth rate for each treatment group, the length of each zebrafish embryo was measured using images acquired at 28 hpf, 48 hpf, and 72 hpf stages and the Adobe Photoshop measurement tool.

4. Gill observation and imaging

The gills of fish that died during the experiment were compared to those of fish in the control bottles. Under a dissecting microscope, using a dissecting knife and forceps, a small piece of skin covering the gills was removed so that images of the gills could be obtained using the MVX10 fluorescent microscope.

RESULTS

1. Effects of oil and dispersant on adult zebrafish

To examine the effects of oil and dispersant on adult zebrafish, fish were exposed to crude oil in the presence or absence of dispersant. Activity levels of the fish in oil, dispersant, and oil/dispersant rapidly declined, relative to the controls. Over time, the fish in the oil/dispersant nearly stopped all movement and remained near the bottom of the bottle (n=4, 20±5 min), where the density of dispersant was the lowest. All of the fish in the oil/dispersant treatment group died within 12 hours post-treatment (hpt). Although the animals treated with oil alone or dispersant alone were also very inactive, they were not fatally damaged, and all survived until the end of the experimental period (24 hpt) (data not shown and Fig. 1).

Microscopic observation of the gills revealed small black particles, which were assumed to be crude oil, present in the fish treated with oil plus dispersant (Fig. 1D). However, no black particles were detected in the same fishes' digestive organs (data not shown), implying that the oil particles broken down by the dispersant had entered the body of the fish

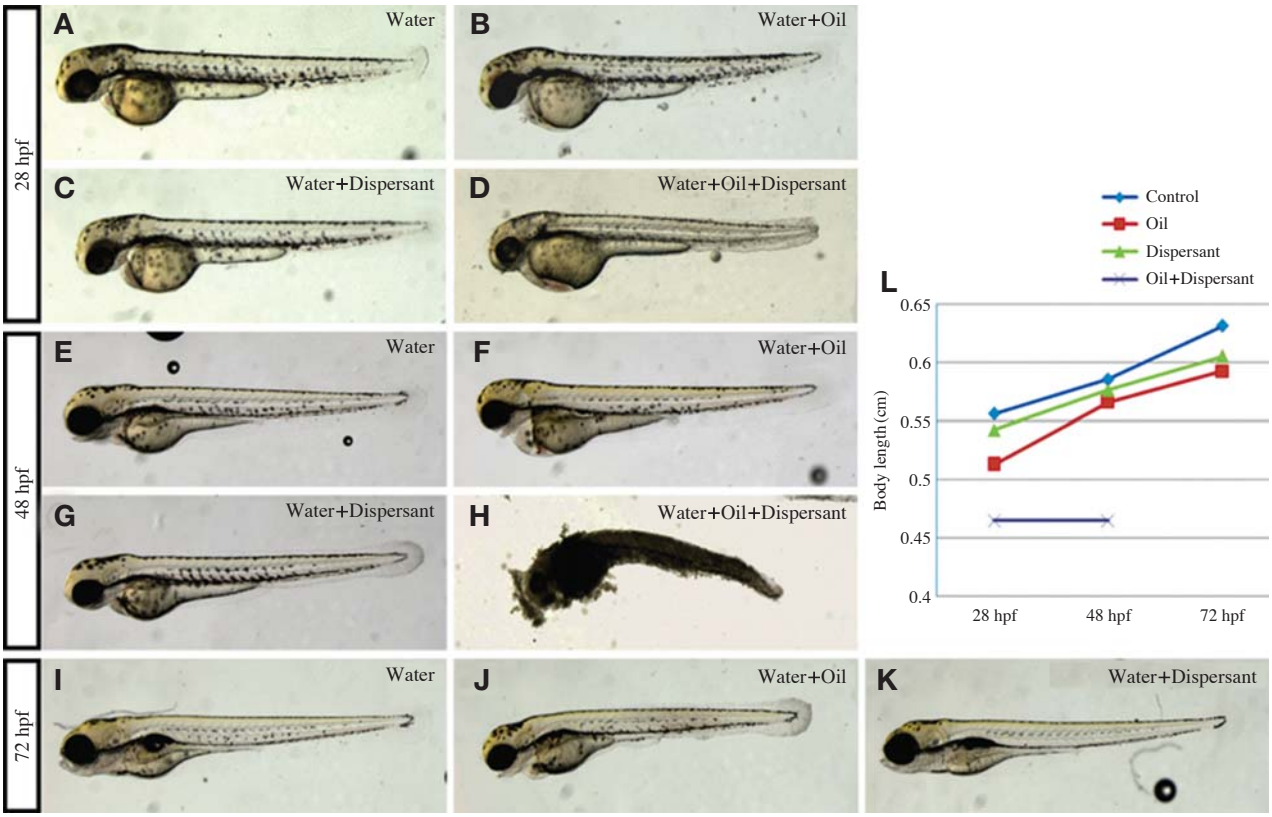

Fig. 2. Effects of oil and dispersant on the development of zebrafish. Images show zebrafish embryos incubated in water alone (A, E, I), treated with oil (B, F, J), treated with dispersant (C, G, K), and treated with oil/dispersant (D, H) at 28 hpf (A～D), 48 hpf (E～H), and 72 hpf (I～K). The embryo in (H) treated with the oil/dispersant combination was dead at 48 hpf. (L) The average length of zebrafish embryos is plotted for each group at 28 hpf, 48 hpf, and 72 hpf (n=50, respectively).

through the gills and/or mouth and interfered with oxygen exchange, ultimately killing them. Thus, oil combined with the dispersant resulted in the most dramatic effects as it killed all the fish overnight. Although the behavior of the fish suggested that oil or dispersant alone was not entirely harmless, the damage resulting from the individual treatments was not as severe as that of the combined treatment.

2. Effect of oil and dispersant on embryonic development of zebrafish

Crude oil and its WAF are known to cause developmental abnormalities, such as edema, in zebrafish embryos (Pauka *et al.* 2011); however, little is known about the effects of dispersant or oil/dispersant combination. To examine the effects of dispersant on zebrafish embryonic development, embryos were exposed to oil with or without dispersant starting at the end of gastrulation (~12 hpf). Compared with the embryos from the control treatment (Fig. 2A), the embryos treated with dispersant, oil, and combined oil with dispersant appeared to have larger yolk sacs (Fig. 2B ~ 2D); however, the differences in body length of each sample at 28 hpf did not reach statistical significance (Fig. 2L). More specifically, at 28 hpf, oil- and dispersant-treated embryos showed normal development in heart, pigment, fin, and eyes (Fig. 2B, 2C). Qualitatively, however, the overall size of the brain and eyes were reduced in oil/dispersant-treated embryos (Fig. 2D).

Embryos treated with oil and dispersant died within ~30 h of treatment, and by the time observations were conducted at 48 hpf, they were already decomposing (Fig. 2H). The other two groups, treated with oil or dispersant alone, were still alive but were less active than the controls, which were swimming freely at 48 h (Fig. 2F, 2G). The embryos treated with oil still had a larger yolk sac at 48 hpf, while embryos treated with dispersant seemed to have a reduced yolk sac which was similar to the size of the controls. Black specks could be seen adhering to the bodies of the embryos treated with oil at 48 hpf. In addition, WAF-treated embryos showed moderate cardiac edema at 48 hpf, as well as yolk sac edema in some fish (n=21, Fig. 2F). In embryos treated with dispersant, the fins had a ragged appearance, unlike the smooth margins present in the controls (Fig. 2G, 2K). The embryos

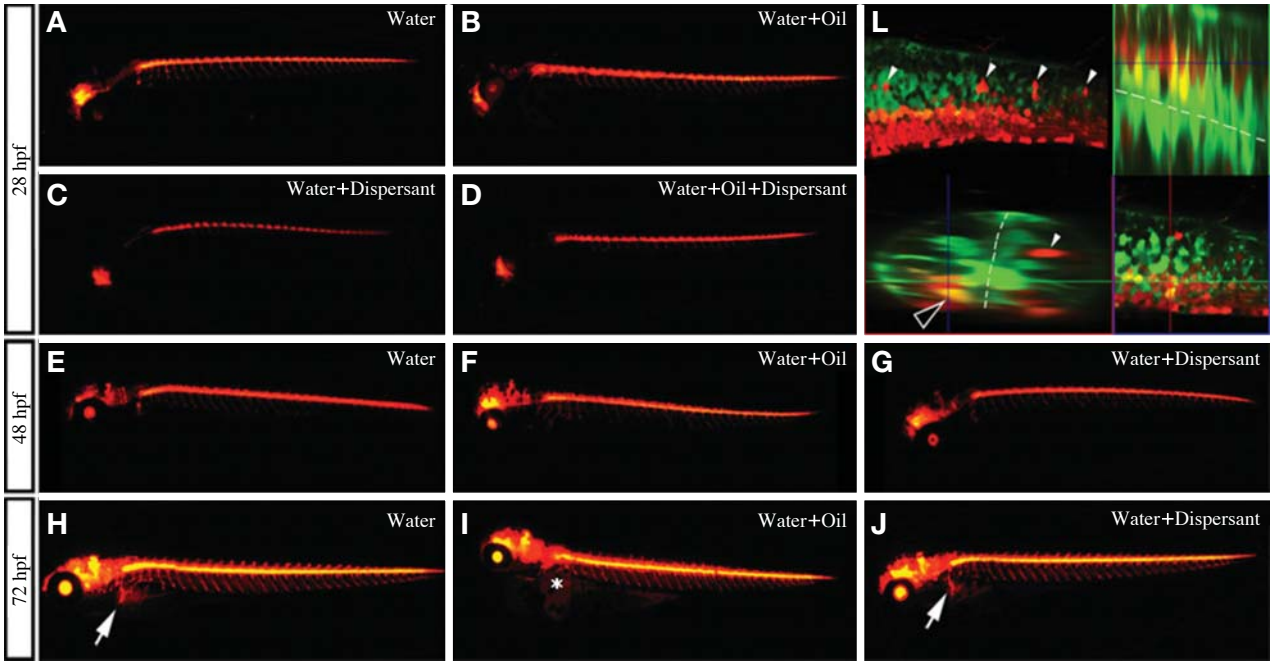

Fig. 3. Effects of oil and dispersant on neural development in zebrafish. Fluorescent images above show a *Tg[olig2-dsRed]* transgenic zebrafish embryos incubated in water alone (A, E, H), treated with oil (B, F, I), treated with dispersant (C, G, J), and treated with oil/dispersant (D) at 28 hpf (A ~ D), 48 hpf (E ~ G), and 72 hpf (H ~ J). Nerves near the pectoral fin (white arrow) could not be detected in the oil-treated embryo (I, asterisk), and were not fully formed in the dispersant-treated embryo (J). Embryos treated with the oil/dispersant combination died. (L) Fluorescent images of the *Tg[Olig2-dsRed]:Tg[her4-GFP]* double transgenic zebrafish embryos showed a motor neuron (open arrowhead) and oligodendrocytes (white arrowheads).

treated with oil or dispersant were inactive and moved very little (Fig. 2J, 2K). Some embryos treated with oil were found dead and decaying (data not shown, n=24), and others suffered from severe heart edema (n=22, Fig. 5B). Only four embryos treated with oil showed moderate abnormality in brain size, which was most apparent in the region of the ventral jaw structures (Fig. 2J).

Zebrafish embryos treated with dispersant appeared to have a faster growth rate than those treated with oil. Zebrafish treated with the oil/dispersant combination exhibited no detectable growth and had a short life span (Fig. 2L). Although we were unable to exclude the possibility that the dispersant alone causes abnormal development, larger yolk sacs, and decreased motility after several days of treatment suggested that the oil/dispersant combination inhibited growth and killed the fish rapidly. Therefore, our data strongly suggest that we all have a moral responsibility to prevent the usage of this dispersant in the area of an oil spill.

3. Effects of oil and dispersant on the neural development of zebrafish

We demonstrated that embryos treated with an oil/dispersant combination used in the Taean spill area exhibited edema, suggesting that usage of dispersant might be detrimental (Fig. 2). Using fluorescent reporter transgenic zebrafish, we next assessed the toxicities of oil and dispersant at the cellular level.

Although the embryos treated with dispersant and with the oil/dispersant combination showed growth delay compared to controls at 28 hpf (Fig. 3A～3D), the changes observed in motor axon development at 28 hpf were insignificant (Fig. 3B～3D). At 48 hpf, we also observed an insignificant change of the motor axon development in the embryos treated with dispersant or oil alone (Fig. 3F, 3G). In the oil/dispersant-treated embryos, it was very hard to analyze changes in the red fluorescence because the majority of the embryos had

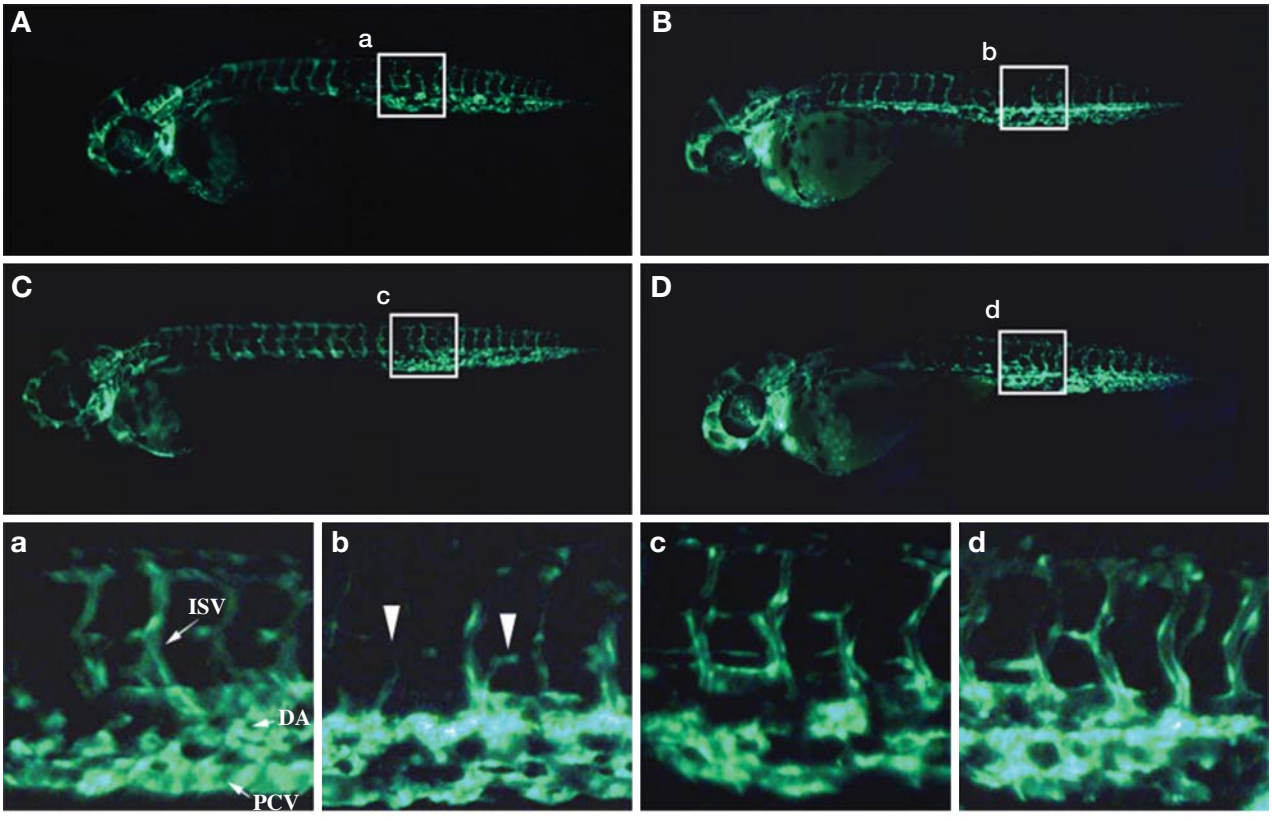

Fig. 4. Evaluating the effect of oil and dispersant on blood vessel development in zebrafish. Fluorescent images show *Tg[flk1-GFP]* transgenic zebrafish embryos incubated in water alone (A), treated with oil (B), treated with dispersant (C), and treated with oil/dispersant (D) at 28 hpf. Figures a, b, c and d show enlarged images of intersomitic vessels, the dorsal aorta, and the posterior cardinal vein. The oil-treated embryo shows defects in ISV development (white arrowheads in b).

already died and decomposed, (data not shown). At 72 hpf, the motor axons toward the pectoral fin were nearly undetectable in the oil-treated embryos (Fig. 3I), and were poorly developed in the dispersant-treated embryos (Fig. 3J). Although it is hard to think of oil itself stimulating production of a repulsive signal against a motor axon from the pectoral fin, and generation of a retention signal in the axon of a motor neuron, the abnormalities of cranio-facial development and cardiac edema might accompany defects of axonal guidance of motor neurons.

4. Effects of oil and dispersant on blood vessel development in zebrafish

At 28 hpf, the predominant difference between oil- and/or dispersant-treated embryos was the development of the intersomitic vessels (ISV) (Fig. 4). The oil-treated embryos showed a lack or defect in the development of the ISV, compared to control embryos (Fig. 4A, 4B). Embryos treated with dispersant alone or oil/dispersant combination appeared to have the least damage (Fig. 4C, 4D), whereas embryos treated with oil alone showed more serious defects at 28 hpf (Fig. 4B). In particular, in the oil-treated embryos, the ISV was hardly visible (Fig. 4b). The longitudinal trunk axial vessels (DA, dorsal aorta; PCV, posterior cardinal vein) were much thinner in the oil-treated embryos than in the controls at 28 hpf (Fig. 4B).

Additional defects were found in the blood vessels of zebrafish embryos at 72 hpf (Fig. 5). The hearts of the oil-treated embryos had abnormally developed into a tube-like shape and developed an edema (Fig. 5B). The ISV were invisible in the oil-treated embryos, indicating abnormal development (Fig. 5B). Development of blood vessels in the brain was also severely reduced (Fig. 5B). Whether the oil directly inhibited the development of the blood vessels or the defects in nearby organs brought about the abnormal development of the blood vessels remains to be determined. Regardless, the defects in the zebrafish embryos caused by oil are very serious. These results indicate that oil plays a major role in forming defects in the brain vasculogenesis,

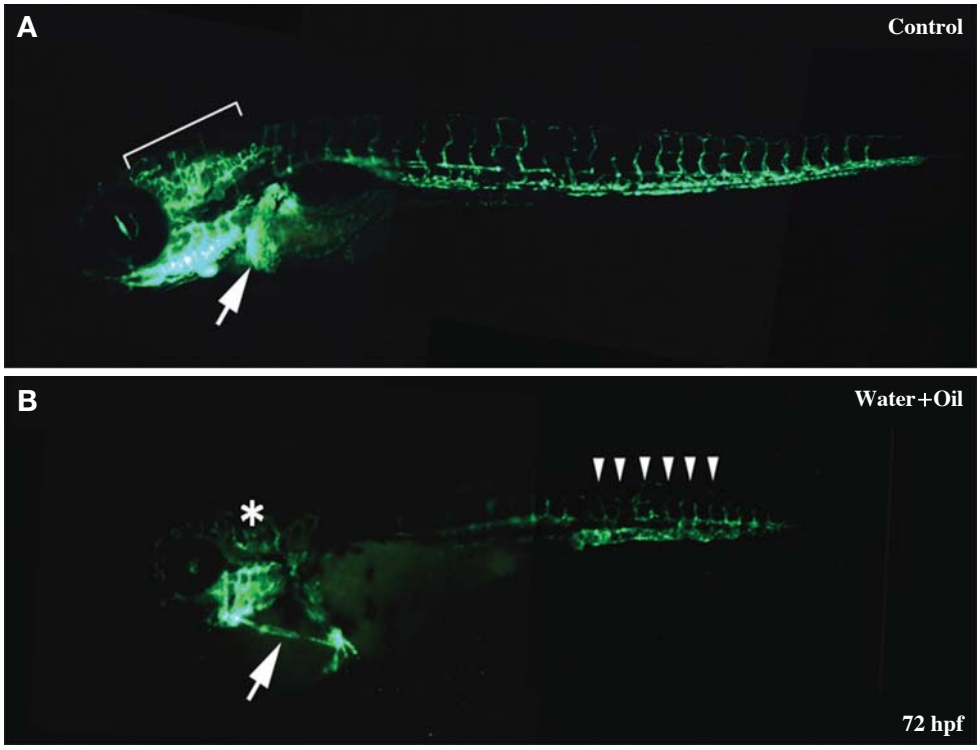

Fig. 5. Oil exposure affects angiogenesis in zebrafish. Fluorescent images show a *Tg[flk1-GFP]* transgenic zebrafish embryo incubated in water alone (A) and an embryo treated with oil (B) at 72 hpf. White arrows indicate the heart and the bracket indicates blood vessels in the brain (A). The oil-treated embryo has abnormalities in brain vessels (asterisk) and ISV (arrowheads). In the oil-treated embryo, edema was present in the heart (arrow in B).

ISV, DA, and PCV, as well as in heart morphogenesis.

DISCUSSION

Once an oil spill occurs, it is very difficult to restore a site to its original condition. Even 20 years after a cleanup, oil can be still at sites of past oil spills. Therefore, the first and foremost precaution is to prevent oil spills from happening at all. Requiring all oil carriers to be constructed as double-hulled is one possibility. There are also patented devices that prevent oil spilling from a ship, and more use should be made of these. Certainly, the world's nations should take part in designing effective and economically feasible strategies and implementing legislation to prevent and deal with oil spills. After the infamous Exxon Valdez oil spill, the US passed a law stating that only double-hulled ships can carry oil in US waters (Ramseur 2012). Passing such laws and providing better management when a spill occurs are needed in every country as the damage from an oil spill can be extensive, and every precaution should be taken to prevent spills (Ramseur 2012).

Using too much dispersant in an effort to rapidly clean up an oil spill site has negative environmental effects (Michel *et al*. 2010). Therefore, alternative methods for cleaning up oil spills must be found. Although progress may be slow, manpower to physically clean an oil spill by hand is one way to remove the oil without causing further damage. Using oil fences to prevent the spread of oil via waves and using sorbents to remove the oil should be considered.

Alternatively, a more environmental-friendly dispersant could be developed (Michel *et al*. 2010). Dispersants break up aggregates of the oil and form micelles in which the hydrophilic region of the dispersant is on the outer surface. When the micelles are formed they may sink to the substratum and create problems. To minimize the damage caused by the dispersant, the micelles should be lighter and smaller to prevent sinking. To accomplish this, the hydrophilic parts should make up a larger portion of the molecule and the molecules should be smaller than those of the currently available dispersants. If possible, ways of making the micelles actually float on the water can be useful, since UV rays from the sun may then naturally break down the micelles.

The experiments presented herein have demonstrated that although oil causes serious damage to vertebrate marine life, the dispersants used to clean it up is even more dangerous. While neither the oil alone nor dispersant alone caused immediate severe damage, the oil/dispersant combination caused rapid lethality and produced remarkable detrimental effects on development and activity. The traditional usage of zebrafish as a tool for toxicology studies has been further enhanced by new and improved techniques for creating stable transgenic fish. For example, genes that are expressed in tissue-specific manner can now be assessed for disruption after chemical exposure by performing whole-mount *in situ* hybridization. Moreover, the laborious staining methods themselves, such as whole-mount *in situ* hybridization, to assess the spatio-temporal expression pattern of a marker gene, have been further improved upon by using a line of zebrafish that has been created to express a transgene with a readily assessable fluorescent reporter, such as GFP. Using this strategy, large-scale chemical screenings can be performed using zebrafish to investigate the effects of various hazardous materials at different developmental stages and test novel targeted therapeutics. In the current study, experiments using *Tg[olig2-DsRed]* and *Tg[flk1-GFP]* transgenic fish revealed that exposure to oil slows down development and causes various defects in the development of nerves and blood vessels. The oil/dispersant combination caused similar developmental defects. It is possible that these observations reflect the actual situation with animals living in a marine site affected by an oil spill and managed with dispersant, such as the site of 2007 oil spill in Korea.

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