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Pannexin channel 1, P2×7 receptors, and Dimethyl Sulfoxide mediate pain responses in zebrafish

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ABSTRACT

The zebrafish has been considered an ideal model for studies of complex behaviors since its behavioral repertoire is well described. Therefore, this study evaluated the perceived pain through behavioral changes in zebrafish larvae. Here we investigated the Acetic Acid (AA) effects on zebrafish larvae exposed in a short-time period (60 s) and the preventive effect from routinely used compounds, Dimethyl Sulfoxide (DMSO), Ethanol (EtOH), Ibuprofen (IBP), and Paracetamol (PAR). In addition, the effect of P2×7 antagonist, A740003, and pannexin channel 1 (PANX-1) inhibitor Probenecid (PROB) on AA-induced behavioral changes were evaluated. AA impaired the distance covered, acceleration, movement, and latency to the first entry in the center from 5 dpf exposed larvae. At 0.050% AA, PAR prevented alterations from the distance covered, acceleration, and movement. Surprisingly, 0.3% DMSO prevented behavioral changes induced by AA. However, the effects from 0.2% DMSO were not prominent. We used 0.2% DMSO as a PROB diluent. PROB prevented the changes in distance and movement observed at both AA concentrations (0.0025% and 0.05%) tested. Since EtOH had no analgesic properties, we used it as an A740003 vehicle to observe the analgesic effects of this compound. As noted, A740003 did not prevent the behavioral changes in the AA-induced pain model. In contrast, 0.2% DMSO and PROB prevented AA-induced behavioral changes. These data enforce that zebrafish could be used in translational studies since this species has behavioral responses related to pain in the early stages of development and responses to analgesics similar to observed in mammals.

1. Introduction

Robust tools have been developing around zebrafish and this animal model has gained space for presenting originality for brain disorders studies [1–3]. The high molecular similarity with humans is no coincidence. Zebrafish responds in a molecularly similar way related to pathologies [4]. Drugs commonly applied to prevent pain and inflammation used in other species show resembling responses in zebrafish [5–7].

Although fish cannot verbalize feelings of pain and discomfort, they do exhibit specific reactions through behavior. Pain mechanisms present some broad features, such as neuropathic pain caused by disease or system damage and nociceptive pain caused by tissue damage. In some cases, both can occur in the same organism [8–10]. Nociceptive pain is one of the ways to study pain in fish. Studies have demonstrated nociceptive responses in fish exposed to several compounds or extreme environments such as Acetic Acid and/or high/low temperature [11–13]. Zebrafish respond well to drugs with analgesic and anti-inflammatory properties, such as Morphine and Diclofenac, that prevented effects caused by AA injection [14]. Exposure to Paracetamol (PAR) did not show behavioral effects in zebrafish, suggesting that it is a safe drug for this species [15].

In fish, nociceptive processing takes place in the forebrain, which includes the telencephalon and diencephalon, which is divided into the epithalamus, thalamus, and hypothalamus. Painful stimuli (mechanical, chemical, or physical) are captured by nerve endings of primary

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neurons, transmitted to peripheral nerves, spinal nerves, and sent to the thalamus through the spinal cord [16]. Therefore, understanding the phenomenon that transforms the painful stimulus into a nervous impulse is essential, since the modulation or alteration of these pathways tend to generate changes in pain responses [17]. The nociceptive stimulus generates an action potential in the axon, which leads to depolarization of the presynaptic membrane. This depolarization causes the opening of the voltage-dependent Ca^{2+} channels. The increasing intracellular Ca^{2+} concentration induces the anchorage and fusion of neurotransmitter vesicles in the presynaptic membrane [18]. This change in intracellular Ca^{2+} concentration also stimulates the opening of Pannexin-1 (PANX-1) channels. These channels are non-selective and form large pores in the plasma membrane [19]. From the opening of PANX-1, the essential nucleotides for intercellular communication, such as ATP, are released into the extracellular environment [20]. The voltage dependence of PANX-1, which is evident in response to change in intracellular Ca^{2+} concentration, ensures that PANX-1 remains closed at the resting membrane potential, with a safety margin against depolarization [21]. The role of PANX-1 in neuronal excitability is mediated by the release of ATP and activation of receptors in the purinergic system.

The nociception mechanisms have, among several pathways, the purinergic system [22]. The purinergic system is a communication path between cells and a major influencer in the transmission of vertebrates pain/inflammatory process, combined in neural and non-neural mechanisms [22–24]. Purinergic signaling is mediated by two families of receptors: P1R and P2R [25–27]. Among these receptors, there is the P2×7, an ionotropic receptor of the P2R family as an ATP-gated ion channel, whose activation results in the opening of channels and the release of pro-inflammatory cytokines, which induces and prolongs the inflammatory process [24]. Among several P2×7 receptor antagonists, the A740003 works well in the zebrafish model [28]. The role of P2×7 in the pain and inflammation process is well described for mammals [29, 30] but remains unclear in the fish pain model. P2×7 receptor interacts with PANX-1 [31] and acts as an ATP-gated cation channel and as a PANX-1 opener to form large pores with high permeability to molecules up to 900 Da [30,32]. Probenecid (PROB) is considered a competitive inhibitor of active transport processes in the brain PANX-1 channel [33]. PROB reversed the zebrafish inflammation induced by copper. However, the A740003 did not reverse the copper effects [31].

To prepare the PROB it was used Dimethyl Sulfoxide (DMSO), a routine compound commonly applied to drug dilution for biological

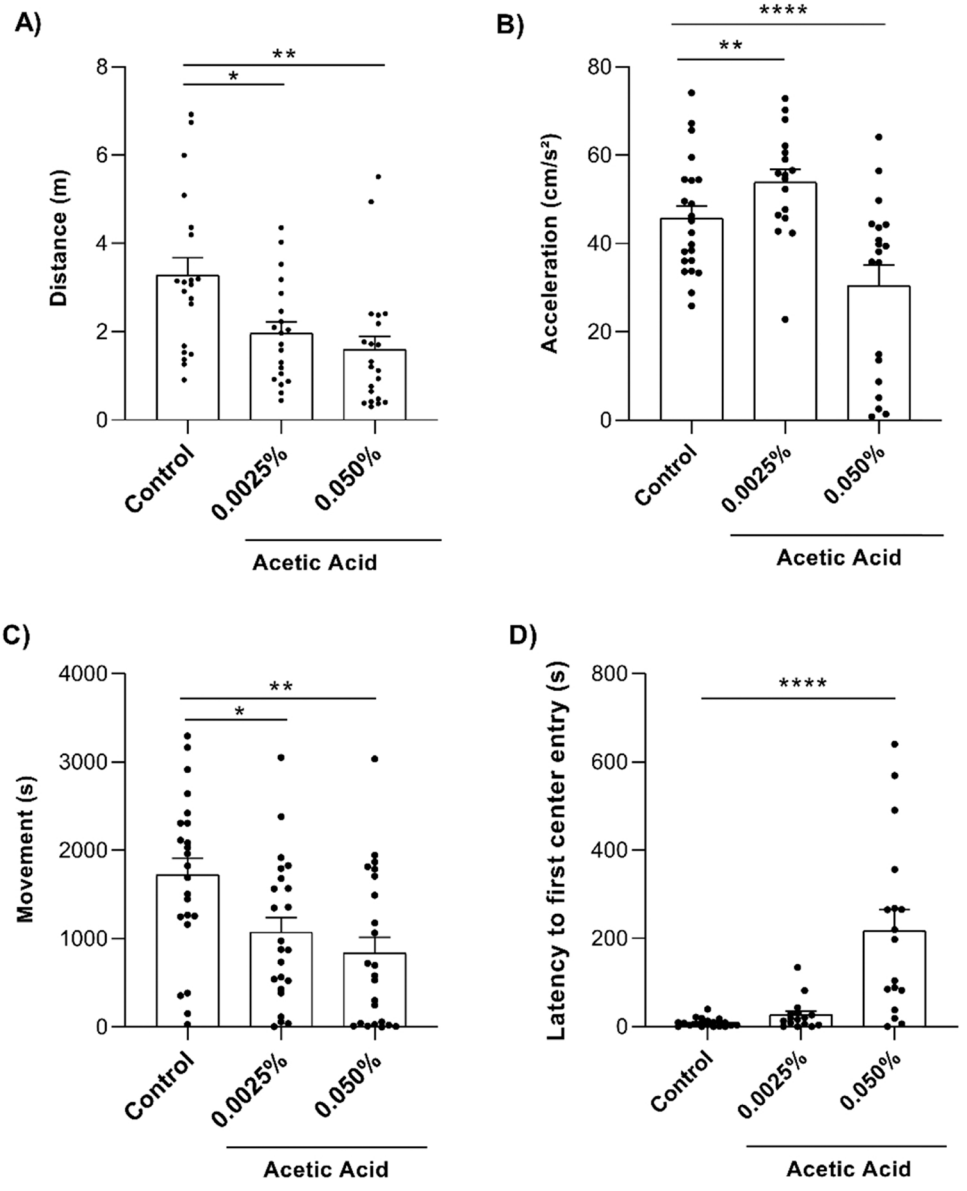

Fig. 1. Locomotor and exploratory behavior was evaluated in 5 dpf zebrafish. Distance (a), acceleration (b), movement (c), and latency to the first entry in the center zone (d) were considered the main parameters to assess the pain response (n = 16–23). Fig. 1A, B, and C were analyzed using one-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. Fig. 1D was analyzed by the Kruskal-Wallis following Dunn's multiple comparisons test. Data are presented as mean ± SEM. * indicates significant difference at $p \leq 0.05$, * * $p \leq 0.001$, and * ** * $p \leq 0.0001$ when compared to the control group.

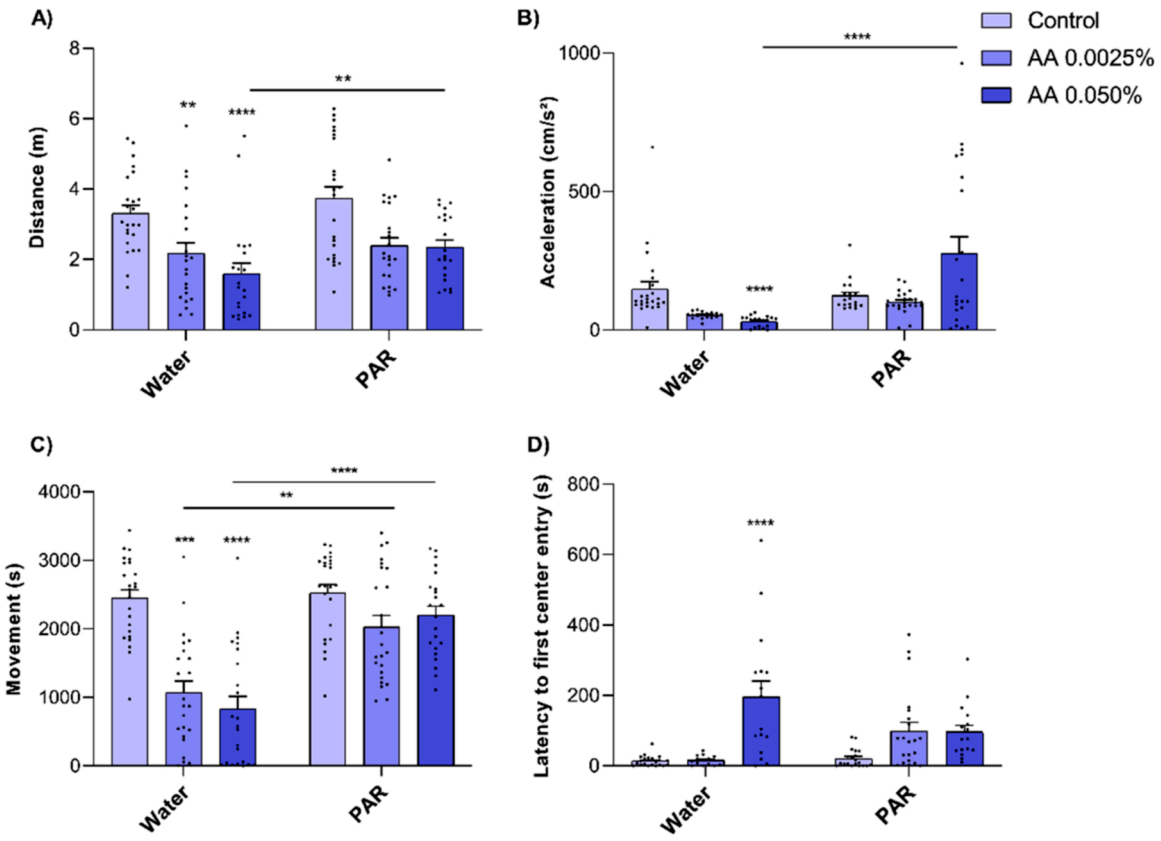

Fig. 2. Distance (a), acceleration (b), movement (c), and latency to the first entry in the center zone (d) were considered the main parameters to assess the preventive PAR effects on pain responses (n = 15–24). Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc* * indicates significant difference at $p \leq 0.05$, * * $p \leq 0.01$, * ** $p \leq 0.005$, and * ** * $p \leq 0.0001$.

assays [31,34]. The DMSO is safe for zebrafish behavior in low concentrations [35]. Several *in vivo* trials have been using DMSO as a vehicle to facilitate drug exposure, *i.e.* zebrafish are constantly exposed to low concentrations [36]. On the other hand, intraperitoneal injections also often have DMSO as a vehicle [37,38]. The safe concentration applied for larvae must not exceed 0.5% DMSO since this concentration did not affect behavioral responses [35]. Despite DMSO has been attributed as a dilution vehicle, analgesic and anti-inflammatory properties were described [39]. Although DMSO is a safe diluting agent, we used Ethanol (EtOH) to dilute A740003. EtOH is an established compound for safe use in animals as a diluent. At low concentrations, EtOH has no direct effects on pain prevention. EtOH showed no effects on behavior at < 0.1% in zebrafish larvae [40], suggesting be safe for the zebrafish model.

The crucial role in behavioral responses to pain remains unclear in fish. To determine the effect of AA on zebrafish larvae, the behavior was studied. Moreover, we have tested the effectiveness of preventing pain from compounds traditionally used in human and veterinary medicine, Ibuprofen (IBP), Paracetamol (PAR), and chemical solvents, such as Dimethyl sulfoxide (DMSO) and Ethanol (EtOH). Furthermore, A740003, a P2×7 receptor antagonist, and PROB, a PANX-1 channel inhibitor, were investigated to clarify a possible preventive-pain role in zebrafish larvae.

2. Methods

2.1. Zebrafish maintenance

All larvae were raised from a core zebrafish facility following established practices. Zebrafish larvae (*Danio rerio*), wild type (AB strain) was used. Each plate (9×9 cm) sustained 20 larvae until 5 days post-fertilization (dpf) with 30 mL water. For the experiment, larvae were caught randomly. The progenitors to generate larvae are maintained in an integrated aquarium system (Zebtec, Tecniplast®, Italy). The Zebtec contains reverse osmosis filtered water at the recommended temperature (28 °C ± 2 °C), pH (7.0–7.5), conductivity (300–700 μS), hardness (80–300 mg/L), ammonia, nitrite, nitrate, and chloride levels for this species. The photoperiod was 14 h light: 10 h dark. Animal's diet was based on feeding with commercial flake and artemia [41,42]. A greenhouse B.O.D (Biochemical Oxygen Demand) with temperature and as standard photoperiod was used for larvae maintenance. All protocols were approved by the Institutional Animal Care Committee (CEUA: 8950, 2018) and followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH). This study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado-SISGEN (Protocol No. A3B073D).

2.2. Drugs exposure

The exposure of the larvae was carried out as follows. First, 5 dpf larvae were exposed to the following compounds for 1 h in Petri dishes and the concentrations were based on previous studies: PAR (0.05 mg/L - CAS number: 103-90-2), IBP (0.005 mg/L - CAS number: 15687-27-1); [15], A740003 (0.1 mM - CAS number: 861393-28-4); [31], PROB (0.1 mM - CAS number: 57-66-9); [31] and DMSO (0.2% and 0.3% - CAS number: 67-68-5). DMSO (0.2%) was used as PROB diluent [31,34] and EtOH (0.1%) was used as A740003 diluent. [40]. The PROB was not tested with EtOH since the final concentration exceeds 0.1% EtOH. Immediately after the drug exposure, larvae were removed and exposed to 0.0025% or 0.050% AA (CAS number: 64-19-7) [13] for 1 min in Petri dishes and straight away after, behavioral analysis were conducted.

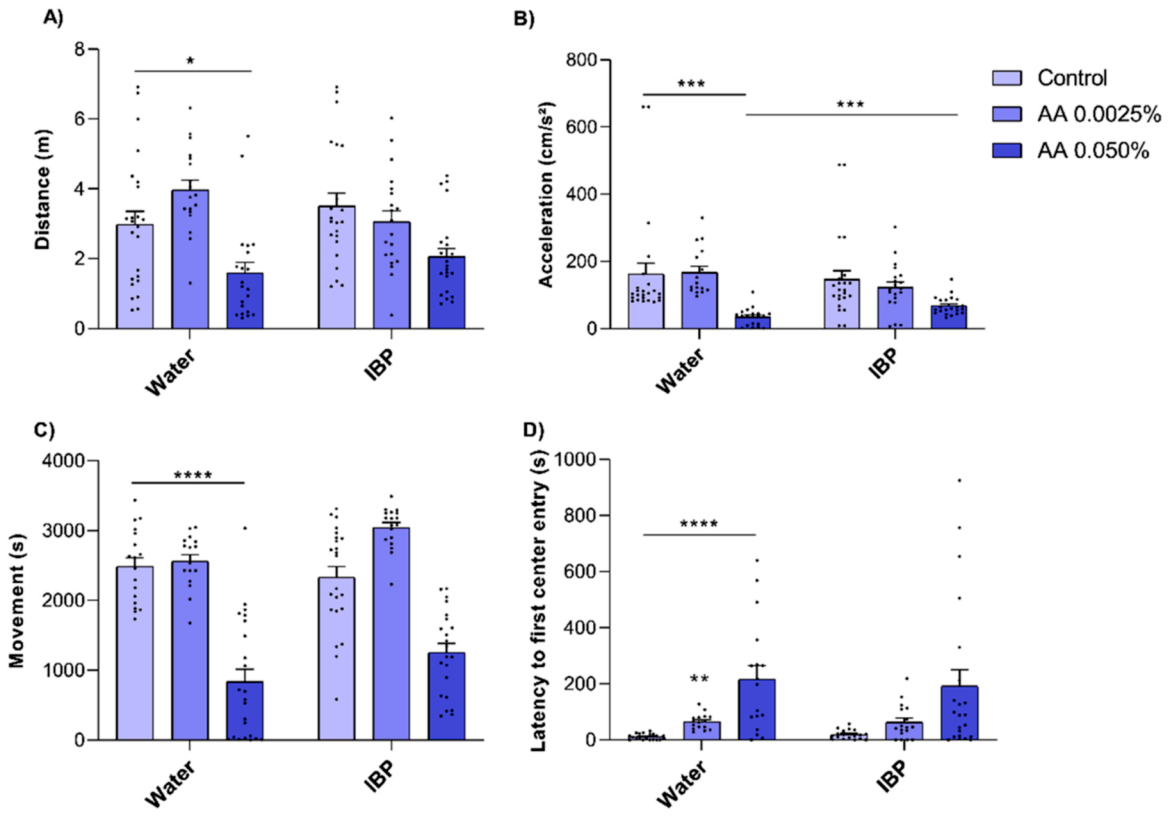

Fig. 3. Distance (a), acceleration (b), movement (c), and latency to the first entry to the center zone (d) were considered the main parameters to assess the preventive IBP effects on pain responses (n = 15–24). Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. * indicates significant difference at $p \leq 0.05$, * **$p \leq 0.005$, and * ** * $p \leq 0.0001$.

2.3. Exploratory behavior

Locomotor activity was assessed at 5 dpf. From each group, larvae were selected and transferred into a 24-well plate with one larva per well, containing 3 mL of system water at 28 ± 2 °C. Larvae were recorded for 60 min following 1 min acclimatization. The records were performed by a tracking device (Noldus Information Technology, Wageningen, Netherlands). Zebrafish pain through exploratory behavior test was assessed by distance covered (m), movement (s), acceleration (cm/s^2) and, latency to the first center entry (s). All data were assessed using EthoVision XT 10.0 Software. A specific parameter movement was previously calibrated to consider the period during which the zebrafish exceeded the start velocity (0.06 cm/s) and remained moving until reaching the stop velocity (0.01 cm/s) [43]. The zebrafish larvae avoid the center of an arena and move towards the periphery of a novel environment [44]. The latency to first entry in the center zone was included to have a more complete scenario of the behavior repertoire in zebrafish larvae exposed to AA.

2.4. Statistical analysis

Normality and distribution were evaluated by the Shapiro-Wilk test. Data were expressed as mean ± standard error of the mean (S.E.M). A significance level of $p < 0.05$ was considered. Fig. 1A, B, and, C were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey test as a *post hoc*. Fig. 1D was analyzed by the Kruskal-Wallis following Dunn's multiple comparisons test. Figs. 2, 3, 4, 5, 6, 7, and 8 were analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. The non-normal data were adjusted through the Log-transformation and analyzed using two-way ANOVA. GraphPad Prism 8 (La Jolla, CA, USA) software was used for statistical analysis.

3. Results

3.1. Acetic acid

Larvae exposed to AA decreased the distance covered (m) in both concentrations tested (0.0025% and 0.050%) ($F_{(2,\ 58)} = 7.342$, $p = 0.0014$; Fig. 1 A). The acceleration increased at 0.0025% AA and contrariwise decreased at 0.050% AA ($F_{(2,\ 55)} = 10.71$, $p = 0.0001$; Fig. 1B). The movement (s) decreased at both AA concentrations ($F_{(2,\ 66)} = 6.464$, $p = 0.0027$; Fig. 1C). At 0.050% AA, larvae take a long time to first entry in the center zone ($H = 21.83$), $p < 0.0001$; Fig. 1D).

3.2. Paracetamol

To investigate the preventive effects from PAR, the larvae were previously exposed for 1 h to PAR and then to AA for 1 min. PAR prevented the distance covered affected by 0.050% AA (AA; $F_{(2,\ 130)} = 18.74$, $p < 0.0001$); (PAR; $F_{(2,\ 130)} = 10.02$, $p = 0.0019$); (Interaction; $F_{(2,\ 130)} = 2.841$, $p = 0.0620$; Fig. 2A) and acceleration (AA; $F_{(2,\ 118)} = 8.676$, $p = 0.0003$); (PAR; $F_{(1,\ 118)} = 20.29$, $p < 0.0001$); (Interaction; $F_{(2,\ 118)} = 10.28$, $p < 0.0001$; Fig. 2B). PAR prevented movement affected by 0.0025% and 0.050% AA (AA; $F_{(2,\ 134)} = 14.28$, $p < 0.0001$); (PAR; $F_{(2,\ 134)} = 34.82$, $p < 0.0001$); (Interaction; $F_{(2,\ 134)} = 10.58$, $p < 0.0001$; Fig. 2C). The latency to first center entry was not prevented by PAR (AA; $F_{(2,\ 89)} = 20.10$, $p < 0.0001$); (PAR; $F_{(1,89)} = 0.6612$, $p = 0.4183$); (Interaction; $F_{(2,\ 89)} = 2.863$, $p = 0.0624$; Fig. 2D).

3.3. Ibuprofen

To investigate the preventive effects from IBP, the larvae were previously exposed for 1 h to IBP and then to AA for 1 min. IBP was not preventive against AA-induced pain in distance covered (AA; $F_{(2,\ 123)}$

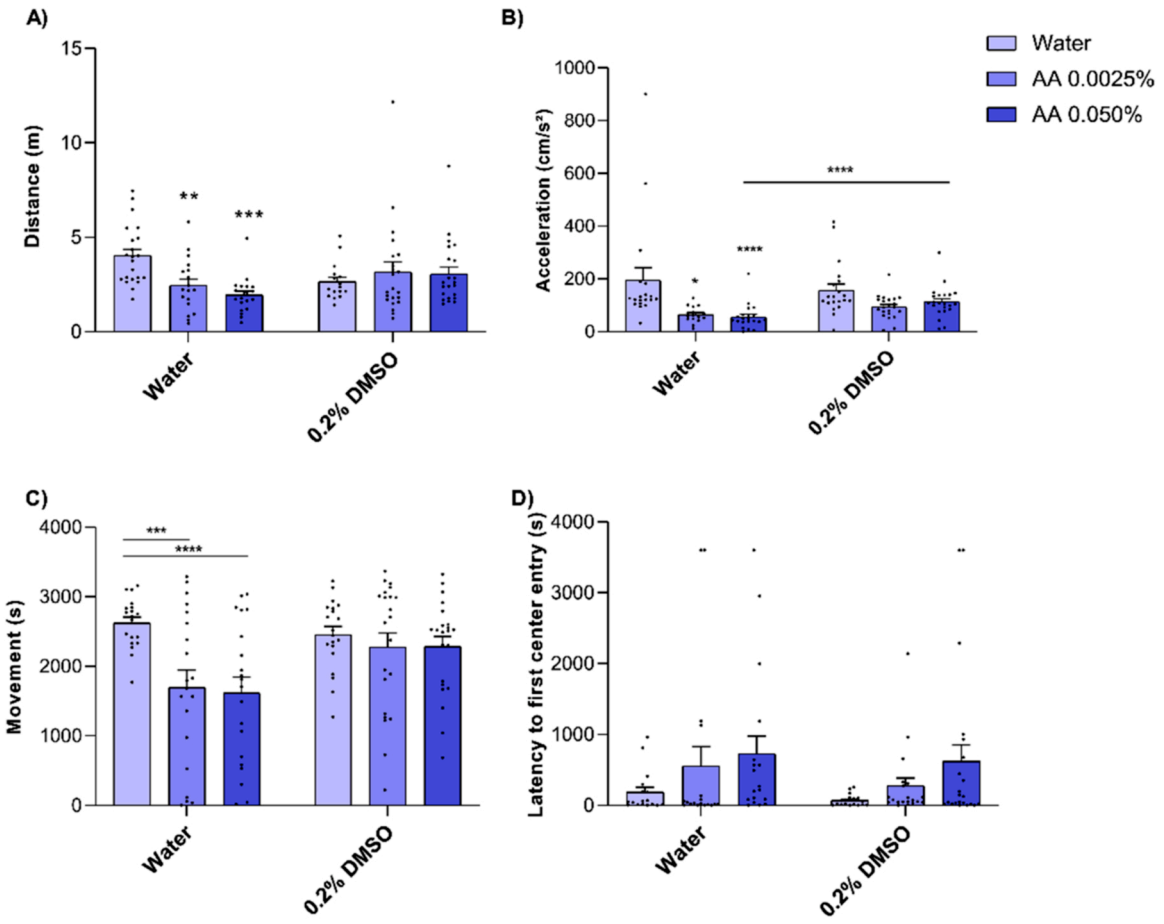

Fig. 4. Distance (a), acceleration (b), movement (c), and latency to the first entry in the center zone (d) were considered the main parameters to assess the preventive effects of 0.2% DMSO from pain responses (n = 16–22). Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. * * indicates significant difference at p ≤ 0.01, * **p ≤ 0.005, and * ** * p ≤ 0.0001.

= 19.34, $p < 0.0001$; IBP, $F_{(1, 123)} = 1.402$, $p = 0.2387$; Interaction, $F_{(2, 123)} = 4.484$, $p = 0.0132$; Fig. 3A), movement (AA, $F_{(2, 114)} = 89.13$, $p < 0.0001$; IBP, $F_{(1, 114)} = 4.713$, $p = 0.0320$; Interaction, $F_{(2, 114)} = 3.099$, $p = 0.0489$; Fig. 3C), and latency to first entry in the center zone (AA, $F_{(2, 94)} = 24.74$, $p < 0.0001$; IBP, $F_{(1, 94)} = 0.5579$, $p = 0.4570$; Interaction; $F_{(2, 94)} = 1.780$, $p = 0.1742$; Fig. 3D). However, the IBP prevented acceleration affected by AA (AA, $F_{(2, 119)} = 25.36$, $p < 0.0001$; IBP; $F_{(1, 119)} = 0.6402$, $p = 0.4252$; Interaction, $F_{(2, 119)} = 10.61$, $p < 0.0001$; Fig. 3B).

3.4. DMSO (0.2% and 0.3%)

DMSO at 0.2% had no preventive effects through distance covered (AA; $F_{(2, 113)} = 4.840$, $p = 0.0096$); (DMSO; $F_{(1, 113)} = 0.9529$, $p = 0.3311$); (Interaction; $F_{(2, 113)} = 6.826$, $p = 0.0016$; Fig. 4A) and latency to first entry into the center zone (AA; $F_{(2, 104)} = 4.784$, $p = 0.0103$); (DMSO; $F_{(1, 104)} = 0.005106$, $p = 0.9432$); (Interaction; $F_{(2, 104)} = 1.237$, $p = 0.2944$; Fig. 4D). It was not also observed preventive effects from movement (AA; $F_{(2, 117)} = 5.011$, $p = 0.0082$); (DMSO; $F_{(1, 117)} = 8.239$, $p = 0.0049$); (Interaction; $F_{(2, 117)} = 2.717$, $p = 0.0702$; Fig. 4C). However, just the acceleration was prevented by DMSO at 0.050% AA (AA; $F_{(2, 113)} = 11.52$, $p < 0.0001$); (DMSO; $F_{(1, 113)} = 5.378$, $p = 0.0222$); (Interaction; $F_{(2, 113)} = 4.481$, $p = 0.0134$; Fig. 4B).

DMSO, at 0.3%, prevented pain effects through distance covered (AA; $F_{(2, 118)} = 8.972$, $p = 0.0002$); (DMSO; $F_{(1, 118)} = 16.47$, $p < 0.0001$); (Interaction; $F_{(2, 118)} = 6.647$, $p = 0.0018$; Fig. 5A) and acceleration (AA; $F_{(2, 113)} = 14.75$, $p < 0.0001$); (DMSO; $F_{(1, 113)} = 25.95$, $p < 0.0001$); (Interaction; $F_{(2, 113)} = 26.07$, $p < 0.0001$; Fig. 5B) at 0.050% AA. Preventive effects through movement (AA; $F_{(2, 129)} = 0.3587$, $p = 0.6993$); (DMSO; $F_{(1, 129)} = 25.75$, $p < 0.0001$); (Interaction; $F_{(2, 129)} = 25.43$, $p < 0.0001$; Fig. 5C) and latency to first entry into the center zone (AA; $F_{(2, 92)} = 1.228$, $p = 0.2976$); (DMSO; $F_{(1, 92)} = 4.109$, $p = 0.0455$); (Interaction; $F_{(2, 92)} = 18.52$, $p < 0.0001$; Fig. 5D) were observed in both AA concentrations.

3.5. Probenecid

The distance covered from PROB (diluted with DMSO 0.2%) exposed animals suggested preventive effects at 0.050% AA (AA; $F_{(2, 98)} = 3.810$, $p = 0.0255$); (PROB/DMSO; $F_{(2, 98)} = 5.307$, $p = 0.0065$); (Interaction; $F_{(4, 98)} = 3.883$, $p = 0.0057$; Fig. 6A). The acceleration was prevented from 0.2% DMSO and PROB at 0.050% AA (AA; $F_{(2, 99)} = 4.067$, $p = 0.0201$); (PROB/DMSO; $F_{(2, 99)} = 15.65$, $p < 0.0001$); (Interaction; $F_{(4, 99)} = 6.390$, $p = 0.0001$; Fig. 6B). However, the preventive effects were more apparent to the movement, where changes induced by both concentrations of AA were prevented by PROB (AA; $F_{(2, 103)} = 11.02$, $p < 0.0001$); (PROB/DMSO; $F_{(2, 103)} = 18.94$, $p < 0.0001$); (Interaction; $F_{(4, 103)} = 3.717$, $p = 0.0072$; Fig. 6C). The latency to first entry into the center did not show alteration by PROB or DMSO (AA; $F_{(2, 94)} = 0.4900$, $p = 0.6142$); (PROB/DMSO; $F_{(2, 94)} = 0.5377$, $p = 0.5859$); (Interaction; $F_{(4, 94)} = 3.578$, $p = 0.0092$; Fig. 6D).

3.6. Ethanol

For ethanol, we did not observe preventive effects in any parameters, such as distance covered (AA; $F_{(2, 90)} = 17.64$, $p < 0.0001$); (EtOH; $F_{(1,}$

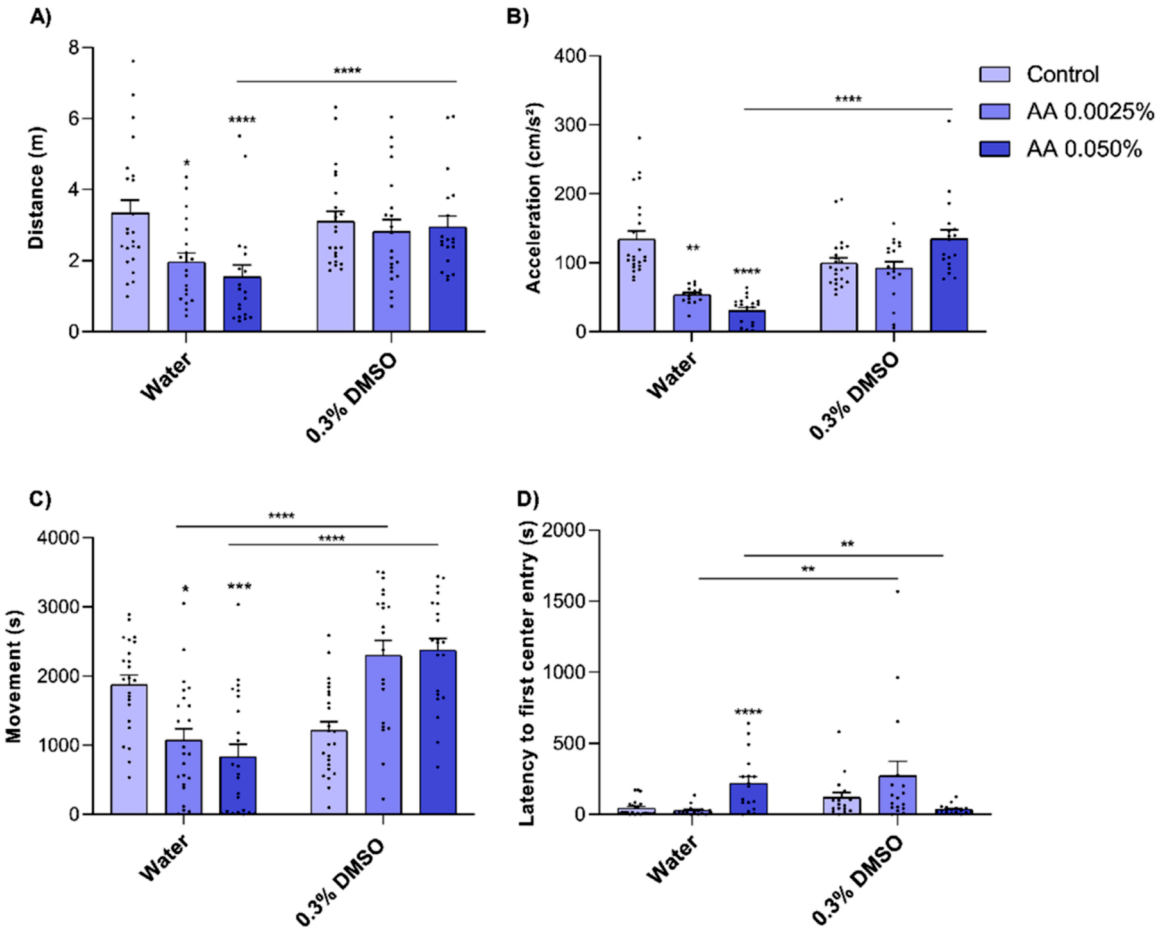

Fig. 5. Distance (a), acceleration (b), movement (c), and latency to the first in center zone (d) were considered the main parameters to assess the preventive effects of 0.3% DMSO on pain responses (n = 19–23). Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. * indicates significant difference at $p \leq 0.05$, * * $p \leq 0.01$, * **$p \leq 0.005$, and * *** * $p \leq 0.0001$.

$_{90)} = 0.3232$, $p = 0.5711$); (Interaction; $F_{(2,\ 90)} = 2.712$, $p = 0.0718$; Fig. 7A), acceleration (AA; $F_{(2,\ 93)} = 15.68$, $p < 0.0001$); (EtOH; $F_{(1,\ 93)} = 2.576e\text{-}005$, $p = 0.9960$); (Interaction; $F_{(2,\ 93)} = 0.01037$, $p = 0.9897$; Fig. 7B), movement (AA; $F_{(2,\ 93)} = 12.21$, $p < 0.0001$); (EtOH; $F_{(1,\ 93)} = 4.979$, $p = 0.0281$); (Interaction; $F_{(2,\ 93)} = 1.156$, $p = 0.3192$; Fig. 7C), and latency to first entry into the center zone (AA; $F_{(2,\ 93)} = 12.21$, $p < 0.0001$); (EtOH; $F_{(1,\ 93)} = 4.979$, $p = 0.0281$); (Interaction; $F_{(2,\ 93)} = 1.156$, $p = 0.3192$; Fig. 7D).

3.7. A740003

The A740003 (diluted with 0.1% EtOH) did not prevent the pain effects in the parameters, such as distance covered (AA; $F_{(2,\ 162)} = 7.066$, $p = 0.0011$); (A740003/EtOH; $F_{(2,\ 162)} = 2.564$, $p = 0.0801$); (Interaction; $F_{(4,\ 162)} = 7.408$, $p < 0.0001$; Fig. 8A), acceleration (AA; $F_{(2,\ 162)} = 7.066$, $p = 0.0011$); (A740003/EtOH; $F_{(2,\ 162)} = 2.564$, $p = 0.0801$); (Interaction; $F_{(4,\ 162)} = 7.408$, $p < 0.0001$; Fig. 8B), movement (AA; $F_{(2,\ 163)} = 7.701$, $p = 0.0006$); (A740003/EtOH; $F_{(2,\ 163)} = 1.037$, $p = 0.3568$); (Interaction; $F_{(4,\ 163)} = 5.947$, $p = 0.0002$; Fig. 8C), and latency to first entry into the center zone (AA; $F_{(2,\ 163)} = 1.845$, $p = 0.1613$); (A740003/EtOH; $F_{(2,\ 163)} = 7.108$, $p = 0.0011$); (Interaction; $F_{(4,\ 163)} = 1.710$, $p = 0.1502$; Fig. 8D).

4. Discussion

The present study investigated specific patterns of pain and its pharmacological modulation to elucidate pain pathways in fish. This study assessed the behavioral repertoire in search of behaviors to clarify the AA effects on the pain/nociception response in zebrafish larvae. After finding specific pattern behaviors attributed to the AA effects, the present study tested the efficiency of drugs, such as PAR and IBP, which are routinely used and have preventive effects already described in zebrafish. In addition, the preventive effects from DMSO and EtOH as well as the PROB and A740003 were also observed.

Here we demonstrated important behavioral data that qualifies distance covered (m), movement (s), acceleration (cm/s^2) and, latency to first center entry (s) as behavioral parameters to identify a pain/nociception model in zebrafish larvae. These behaviors can be considered robust for neuropharmacological analysis in zebrafish [45,46]. Several studies have used AA as pain induction in zebrafish [13,14,47]. In this study, we investigated behavioral parameters and validated them by chemical compounds commonly used due to their analgesic properties.

Several studies using zebrafish larvae as an animal model to evaluate pain responses are focused on the investigation of exploratory behavior, showing the use of different inducers, such as acetic acid, citric acid [48], hypothermia, and hyperthermia [49]. A study conducted by Steenbergen and Bardine (2014) determined that 5dpf larvae submitted to 0.0025%, 0.005%, 0.01%, and 0.025% AA increased the distance covered at all concentrations during 180 s tracking. Our study evaluated behavioral responses one hour after pain induction by the AA. The larvae decreased the distance covered, acceleration, and movement after exposure to 0.0025% and 0.050% AA as well there was an increase in the latency to the first entry in the center zone. The larvae were placed individually in 24-well plates and the exploratory behavior was analyzed. As it is considered a new environment, the tendency is for them to explore as much as possible to form a spatial memory and then,

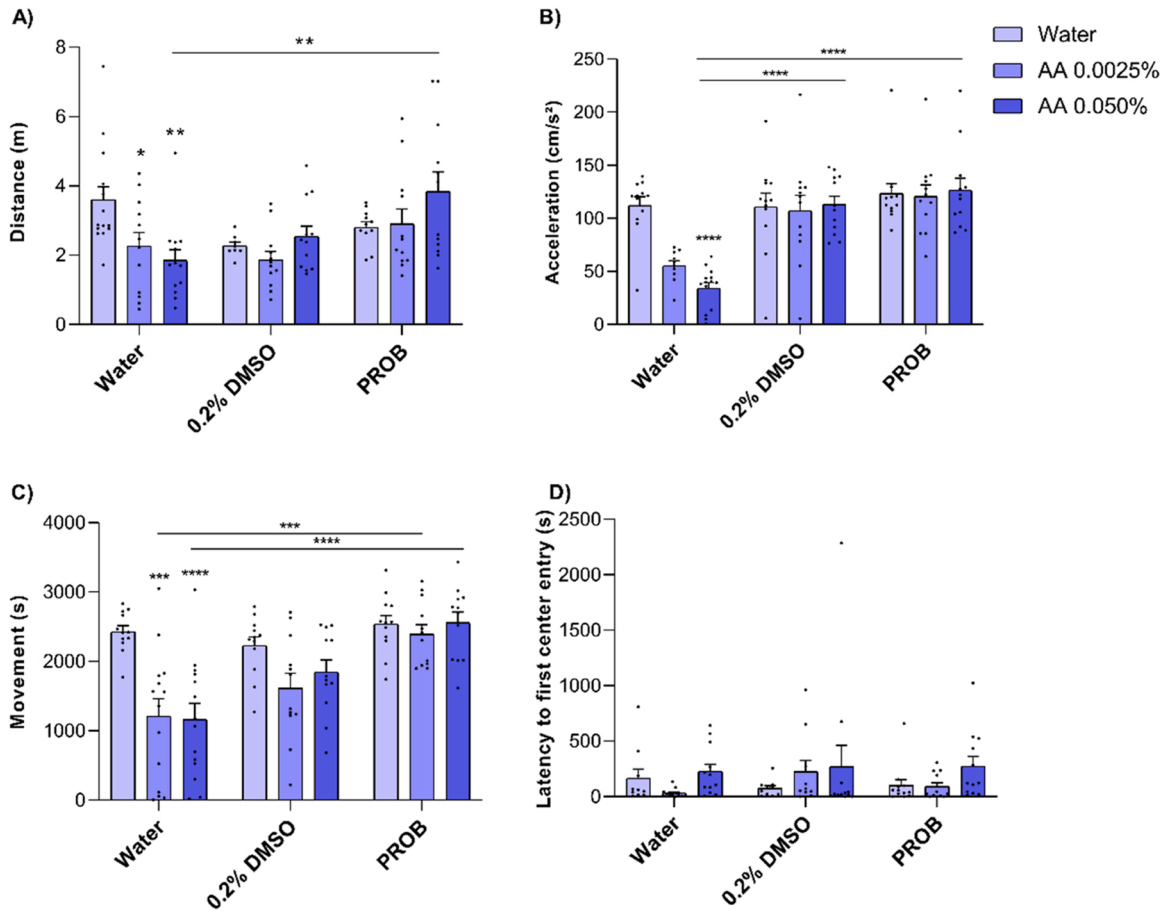

Fig. 6. Distance (a), acceleration (b), movement (c), and latency to the first entry in the center zone (d) were considered the main parameters to assess the preventive effects from 0.2% DMSO or 0.2%DMSO plus 0.1 mM PROB (n = 10–14) on pain responses. Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. * indicates significant difference at $p \leq 0.05$, * * $p \leq 0.01$, * **$p \leq 0.005$, and * ** * $p \leq 0.0001$.

over time, feel safer [43,50]. Usually, this exploration takes place entirely in five minutes whereas they remain immobile for around 60 s [43,50]. We demonstrated that larvae exposed to AA reduced exploratory behavior during one hour of tracking. In addition to causing a pain effect, AA may also be responsible for leading the larvae to an anxiety-like behavior because tissue damage is occurring in relatively high proportions. On the other hand, the 0.0025% AA reduced the pH to 4.59 and at 0.050% to pH 3.4, similar as previously described [13]. The pH would be responsible to impair the behavior [51]; however, we observed that PAR prevented the effects caused by AA. The PAR analgesic properties are known through the inhibition of COX [52] and were described in zebrafish [53] and, as far as we know, do not affect pH change sensation. PAR is used worldwide as a first choice for the treatment of early pain symptoms [54]. The action mechanism of PAR is binding with COX [52], and zebrafish responded very well through this route [53] similarly to mammalians [55]. For a long-lasting time, PAR was described as a COX inhibitor. Although recent studies suggested new binding pathways for PAR, the known potent analgesic effects are not discussed [52,54,56]. Zebrafish larvae showed a change in COX-2 mRNA caused by AA at 0.0025% and 0.025% between ten and thirty minutes after exposure. After thirty minutes, they did not observe changes in COX-2 mRNA [13]. For being a potent analgesic, we observed that PAR was effective in preventing effects caused by AA. A pain/nociceptive stimulus induced by AA acts in the skin by generating a peripheral nerve action potential and carrying that signal through spinal nerves. This stimulus goes to the spinal cord to reach the thalamus. After reaching the thalamus, the telencephalon processes this information for the animal to respond. After AA pain induction, the COX may not catalyze the conversion of arachidonic acid to prostaglandin H2 (PGH2); however, the blockade caused by PAR interrupted the PGH2 biosynthesis effects. The prostaglandins biosynthesis plays a crucial role in pain signaling [57]. Our results have demonstrated that, before the exposure to AA, the larvae remained for one hour immersed in the PAR and showed signs of prevention of pain caused by 0.0025% AA, indicating that PAR promoted analgesic effects in the zebrafish pain model. Unlike PAR, our findings have shown that IBP was not effective in terms of pain prevention in the zebrafish pain model. IBP has strong anti-inflammatory effects and moderate long-lasting analgesic effects. The analgesic effects peak occurred 1–2 h after administration [58,59]. The main mechanism of action of IBP is the inhibition of COX 1 and 2 [60] that was previously described in zebrafish [53]. Analgesia is not as noticeable in IBP as compared to PAR, although IBP has analgesic effects, it has greater applicability as an anti-inflammatory [61,62]. The IBP is considered a drug used with anti-inflammatory properties firstly. Here we demonstrated behavioral effects caused by the AA that was supposed to induce pain/nociception. The IBP effects would be more pronounced considering other parameters involved in inflammatory responses. Unlike adult humans and some animals that can verbalize feelings, fish, as far as we have known, are not capable of screaming indicating feelings of pain, although they respond molecularly in a similar way to humans [4]. As with human babies, the search for behaviors-related to possible sensations of pain is constant [63].

The effects of DMSO, an aprotic organic solvent with high biological membrane penetration and low systemic toxicity, have been studied [64]. DMSO has analgesic and anti-inflammatory properties described [65]. Our findings demonstrated that 0.3% DMSO induced a high

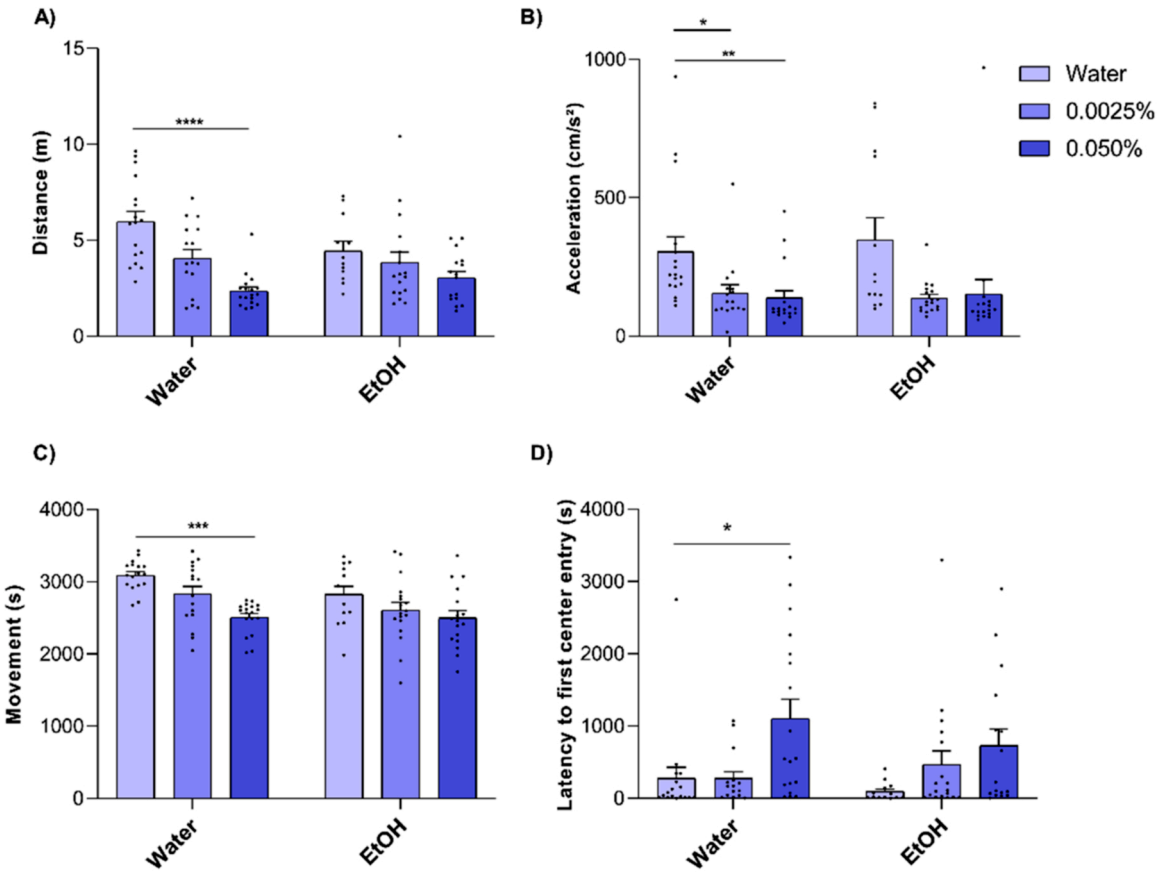

Fig. 7. Distance (a), acceleration (b), movement (c), and latency to the first entry in the center zone (d) were considered the main parameters to assess the preventive effects from EtOH on pain responses (n = 12–18). Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. * indicates significant difference at $p \leq 0.05$, * * $p \leq 0.01$, * **$p \leq 0.005$, and * ** * $p \leq 0.0001$.

movement when compared to AA-treated animals. We found the preventive effects caused by 0.3% DMSO to all behavioral parameters, which were different from 0.2% DMSO that only prevented acceleration. As demonstrated, 0.3% DMSO increased the PANX1a expression [31] and allows ions to enter the cell, which causes cell excitation. DMSO promotes a wide spectrum of pharmacological effects [66]. Since its first use, DMSO has been widely used in the equine industry to reduce soft tissue swelling, inflammation, and edema secondary to acute trauma. The analgesic route of DMSO is through prostaglandins inhibition and is effective against both acute and chronic musculoskeletal pain [67]. Here we demonstrated reduced parameters caused by AA, which 0.3% DMSO was able to reverse, suggesting effects on the prostaglandin pathway [67].

Among several mechanisms involved, the purinergic system is a pathway that may be related to pain mechanisms in fish [22]. Through the PANX channel, several works related a novel way to study neuropathic pain mechanisms [67–70]. Neuropathic pain in rats was reduced by L-kynurenine–probenecid combination [70] as well trigeminal activation-related pain conditions were treated by PROB [69]. Our study demonstrated that DMSO had differing responses, depending on the concentration. Because of the strong effects of 0.3% DMSO on the pain model, we used a lower DMSO concentration (0.2% DMSO) as PROB diluent. We observed that PROB diluted in 0.2% DMSO presented robust preventive effects on pain responses in zebrafish. Even though the DMSO increased the expression of PANX1a [31], the antagonist effect of PROB on the PANX channel was superior. The present data may suggest that PROB, even diluted in 0.2% DMSO may have robust interaction in the model of pain induced by AA. The blockade of PANX prevented effects of distance covered, acceleration, and movement caused by the AA.

Later, for presenting effects on the inflammatory process [22,24,25] and some studies suggested the analgesic properties [30,71], we tested the A740003 effects. A740003 was used as a P2×7 receptor antagonist to elucidate the relationship of this pathway with the AA pain model. We evaluated the effects of A740003 using EtOH as a vehicle. Our findings demonstrated that EtOH did not present analgesic effects. We did not observe any interaction by EtOH alone or EtOH plus A740003. For this reason, we conclude that A740003 has no apparent effects on the AA-induced pain model in zebrafish larvae. However, at low AA concentration, we did observe prevention on distance by A740003. Despite this effect on the distance covered, we cannot affirm the analgesic effects from this compound. The A740003 effects could be apparent if more concentrations were tested and serve as a study limitation to encourage us to analyze these concentrations in future studies.

We also tried to use EtOH as PROB diluent, however, does exceed the maximum concentration (0.1% EtOH), which might interact with the behavior [40]. The 0.1% EtOH was not able to dilute PROB. Further studies are needed to clarify the pathways involved in the analgesic effects by PROB. Several studies have been referring to PANX as a signaling pathway for neuropathic pain [68,72,73], which may demonstrate new analgesic pathways. Over time, exposure to AA can trigger the onset of the inflammation cascade [74]. Although we have investigated pain/nociception, it is possible to suggest that A740003 and PROB could be used in inflammation models in zebrafish [74,75].

In summary, we evaluated a pain/nociception model following AA exposure in a short-time period. First-choice drugs for pain prevention in mammals, such as PAR, prevented pain caused by the AA. In contrast, IBP was not able to prevent pain from the AA-induced pain model in zebrafish. Our data also suggested that DMSO would be a potent analgesic to the zebrafish pain model since 0.3% DMSO showed analgesic effects when compared to 0.2% DMSO. PROB, a PANX-1 inhibitor was

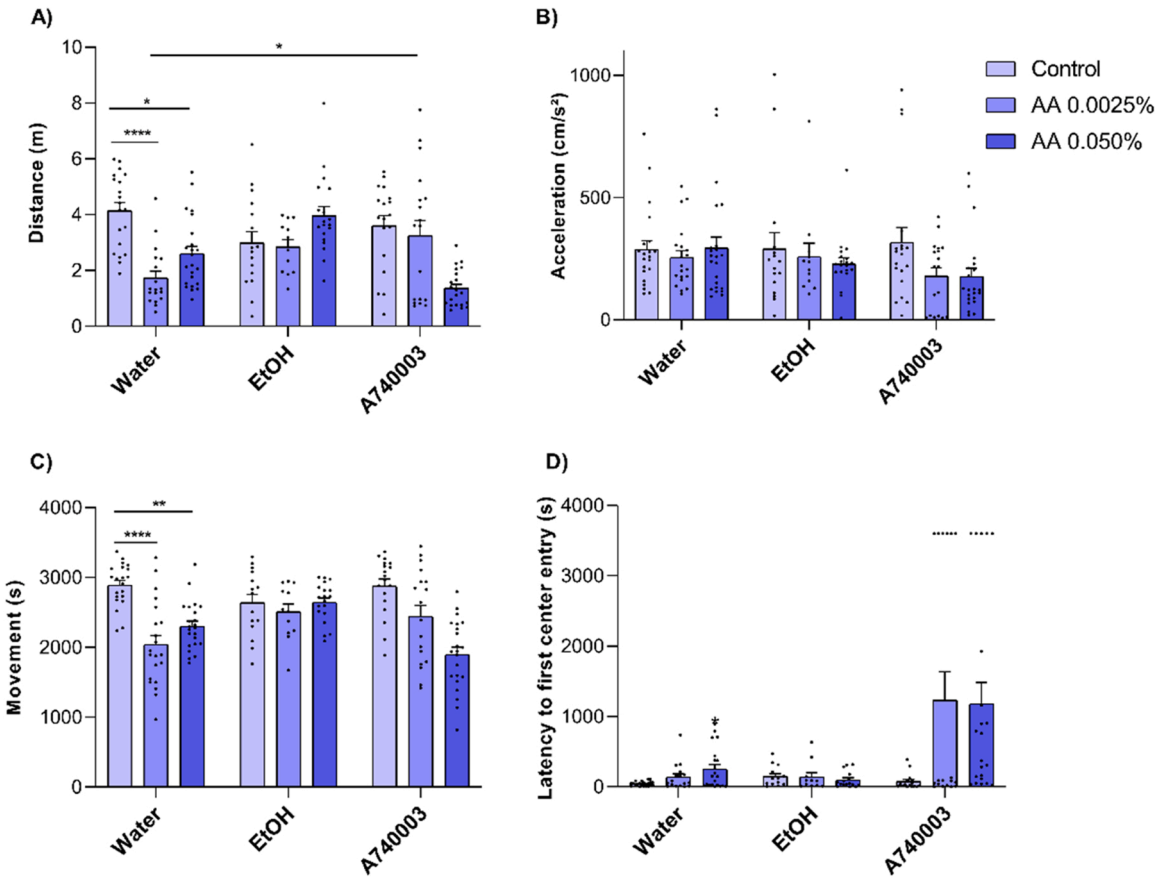

Fig. 8. Distance (a), acceleration (b), movement (c), and latency to the first entry in the center zone (d) were considered the main parameters to assess the preventive effects from EtOH or EtOH + A740003 (n = 12–23) on pain responses. Data are presented as mean ± SEM and analyzed by two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. * indicates significant difference at $p \leq 0.05$, * * $p \leq 0.01$, and * ** * $p \leq 0.0001$.

also effective to prevent pain induced by AA in zebrafish larvae.

CRediT authorship contribution statement

Darlan Gusso: Conceptualization, Investigation, Methodology, Writing – original draft preparation, Data curation. **Fernanda Fernandes Cruz:** Investigation. **Pâmella Moreira Fritsch:** Investigation. **Marília Oberto da Silva Gobbo:** Investigation. **Fernanda Bueno Morrone:** Resources, Supervision, Writing – review & editing. **Carla Denise Bonan:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Conflict of interest

The authors declare that there is no conflict of interest.

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