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P2X7R and PANX-1 channel relevance in a zebrafish larvae copper-induced inflammation model

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ABSTRACT

Copper is a metal that participates in several essential reactions in living organisms, and it has been used as an inflammatory inducing agent in zebrafish larvae. In this study, we evaluated the effect P2X7 receptor and/or pannexin channel 1 (PANX-1) blockage in this inflammation model. To perform the experiments, 7 dpf larvae were exposed to 10 μM of copper and treated with 100 μM probenecid, PANX-1 inhibitor, and/or 300 nM A740003, a P2X7R selective antagonist. Larvae survival was assessed up to 24 h after treatments. The evaluation of larvae behavior was evaluated after acute (4 h) and chronic (24 h) exposure. The parameters of locomotor activity measured were: mobile time, average speed, distance and turn angle. We analyzed the gene expression of the P2X7 receptor, PANX1a and PANX1b channels and interleukins IL-10 and IL-1b after 24 h of treatment. Treatments did not decrease larval survival in the time interval studied. Changes in larvae locomotion were observed after the longest time of exposure to copper and the treatment with probenecid was able to reverse part of the effects caused by copper. No significant difference was observed in the oxidative stress assays and probenecid and copper treatment decrease partially PANX1a gene expression groups. The data presented herein shows the relevance of the blockage of P2X7-PANX-1 in copper-induced inflammation.

1. Introduction

Copper is a metal that participates in several essential reactions in living organisms, but in high concentrations can become toxic (Festa and Thiele, 2011). Copper ions under the Cu^{+} (reduced) or Cu^{2+} (oxidized) states predominate in biological systems, the exchange between these states generates hydroxyl radicals that are important for different enzymatic reactions, however the increased concentrations of these reactive oxygen species (ROS) are involved in the induction of inflammatory processes (Scheiber et al., 2014; Tisato et al., 2009). High levels of redox metals promote the formation of ROS, which will induce an inflammatory process in several models (Choo et al., 2013; Kennedy et al., 1998; Pereira et al., 2016; Schmalz et al., 1998).

The model of copper-induced inflammation in zebrafish is already well established (Alençon et al., 2010; Leite et al., 2013). The previous study showed that zebrafish larvae exposed to copper sulphate ($CuSO_4$) develop a lesion in the neuromast hair cell (Hernández et al., 2006). This lesion is probably related to the production of reactive oxygen species (ROS), generated through the copper reduction process, which is also responsible for promoting an inflammatory process (Brown and Borutaite, 2001; Oyinloye et al., 2015). Recently, some studies have shown the involvement of the purinergic system in this model of inflammation (Cruz et al., 2017; Leite et al., 2013).

Purinergic signaling is mediated through two families of purinergic receptors: P1R (A_1, A_{2A}, A_{2B} and A_3) and P2R, which are subdivided into seven P2X ion channel receptor subtypes (P2X1-7) and eight G protein-coupled receptors subtypes ($P2Y_{1,2,4,6,11-14}$) (Di Virgilio, 2012). These receptors are widely expressed in different mammalian cells mediating a wide variety of biological responses (Burnstock, 2006). The extracellular levels of ATP and adenosine are regulated by the action of ectonucleotidases bounded to the plasma membrane, such as ectonucleoside triphosphate diphosphohydrolase (ENTPDase1/CD39) and ecto-5′-nucleotidase/CD73 (CD73). CD39 efficiently hydrolyzes ATP to ADP and AMP, while CD73 converts AMP to adenosine (Bastid et al.,

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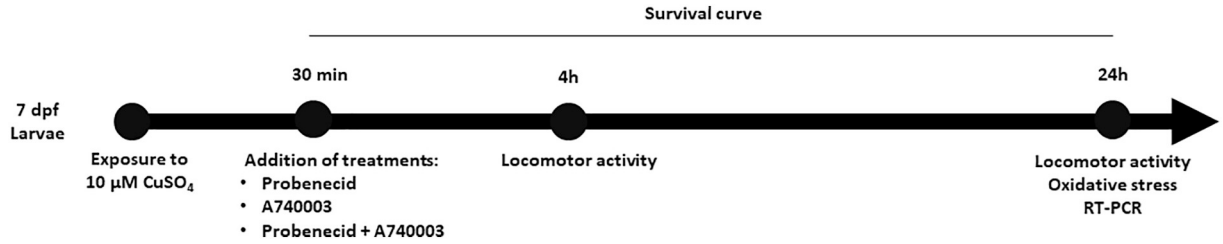

Fig. 1. Experimental scheme of zebrafish exposure to $CuSO_4$ and pharmacological treatments. For induction of inflammation, 7 dpf zebrafish larvae were exposed to 10 µM $CuSO_4$ for 30 min and, posteriorly treated with probenecid and/or A740003. Larvae survival was determined up to 24 h after treatments. Locomotor activity was assessed after 4 and 24 h. Oxidative stress assays and RT-PCR were performed in larvae treated for 24 h.

2013; Zimmermann et al., 2012).

Extracellular nucleotides act as signaling molecules and they have their extracellular concentrations increased after cellular damage (Boeynaems and Communi, 2006; Burnstock, 1972). ATP has been described as an important inflammatory mediator (Junger, 2014) that promote biological effects through its binding to purinergic receptors, especially P2X7R (Burnstock and Knight, 2018). The P2X7R are ionotropic receptors, which are expressed in hematopoietic cells, such as monocytes, macrophages, and lymphocytes (Jacob et al., 2013). Their activation results in the opening of channels and as a consequence occurs the release of pro-inflammatory cytokines, such as IL-1β, TNF, that will induce and perpetuate the inflammatory process (Alves et al., 2013; Sanz and Virgilio, 2000).

Pannexin channels are important in the release of ATP to the extracellular medium (Bao et al., 2004; Cotrina et al., 1998; Penuela et al., 2013). Pannexin channel 1 (PANX-1) is the most studied channel and the reason is due to its frequent interaction with the P2X7R (Alberto et al., 2013; Diezmos et al., 2018; Locovei et al., 2007). Previously, it was observed that there is a physical and functional interaction between P2X7R and PANX-1, which results in IL-1β release, through the formation of a large pore in the cell membrane (Pelegrin and Surprenant, 2006). It is also known that P2X7R- PANX-1 is associated with NLRP3 inflammasome activation in several cell types (Silverman et al., 2009).

Since the P2X7R-PANX-1 interaction was shown to be an important complex in inflammatory processes, this study aimed to evaluate the involvement of the P2X7R and the PANX-1 in a zebrafish larvae copper-induced inflammation model.

2. Materials and methods

2.1. Animals

Zebrafish larvae with 7 dpf (days post-fertilization) were obtained through breeding matrices (zebrafish-AB) maintained in a semi-automated Zebtec system (Tecniplast®, Italy). The animals were mated according to Westerfield (2000) and the obtained embryos were kept in a B.O.D under the standard conditions: light/dark cycle of 14/10 h respectively, and temperature of 25 ± 2 °C. The embryos/larvae were collected and kept in maintenance water, which is supplied by reverse osmosis water and is conditioned with the following parameters: pH 6.5–7.5; conductivity 400–600 µS, and ammonium concentration < 0.004 ppm. All procedures were approved by the Ethics Committee for Animal Use of PUCRS (8039, CEUA-PUCRS).

2.2. Chemicals

Copper, added as copper sulfate pentahydrate ($CuSO_4$·5H2O) was acquired from Merck (Darmstadt, Hessen, Germany), A740003 was acquired from Santa Cruz Biotechnology (Dallas, TX, USA) and probenecid was purchased from Sigma (St. Louis, MO, USA).

2.3. Treatments

Zebrafish larvae were exposed to 10 µM $CuSO_4$ for 30 min, for the absorption of the inflammatory agent and induction of systemic inflammation, as described previously (Alençon et al., 2010; Leite et al., 2013). Posteriorly, pharmacological treatments were added to larvae medium, in order to assess the involvement of the pannexin channel and P2X7R in this inflammatory context. Animals were treated with a P2X7R antagonist, 300 nM A740003 (Cruz et al., 2013), and 100 µM probenecid, a PANX-1 inhibitor (Silverman et al., 2008). The concentration of probenecid was chosen through a survival curve. The animals were divided in groups, which were treated with different combinations of the compounds ($CuSO_4$; A740003; probenecid; $CuSO_4$ + A740003; $CuSO_4$ + probenecid; $CuSO_4$ + A740003 + probenecid). $CuSO_4$ was dissolved in milli-Q water, and A740003 and probenecid were dissolved in DMSO (final concentration of 0.3%). Treatments were executed in 6-well plates with 20 larvae per well. Evaluations were performed at two different periods of exposure, since it was described that $CuSO_4$ can induce different inflammation profiles according to the exposure time: 4 h are representative of the initial phase of the inflammatory process, whereas 24 h of exposure is characteristic of a late stage of inflammation (Leite et al., 2013). The treatment and assessment scheme is described in Fig. 1.

2.4. Survival curve

For the evaluation of animals' survival, it was used 60 larvae per group, which were exposed to treatments for 24 h. The mortality was determined at the time points of 2, 4, 8 and 24 h after treatments. To ensure larvae death, it was observed in a stereomicroscope the parameters of mobility, color, and larvae position. A decrease in survival ≥ 20% was considered as significant.

2.5. Locomotor activity

The locomotor activity was performed after 4 and 24 h of treatment. The larvae were placed individually per well in a 24-well plate containing system water (drug-free) with a 1 cm column. According to Colwill and Creton (2011), larvae locomotion was recorded for 5 min with 1 min of acclimatization in the apparatus. The recorded videos were analyzed by Any-Maze Version 6.06 tracking software. The parameters evaluated were: distance (m), mobile time (s), turn angle (°) and velocity average (m/s). To measure larvae velocity, it was considered the distance and the mobile time. This assay was performed using at least 12 larvae per group (Capiotti et al., 2011; Cruz et al., 2017).

2.6. RNA isolation and real-time RT-qPCR

Molecular analysis of gene expression was performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines for RT-qPCR experiments (Bustin et al.,

2013, 2009). The total RNA was isolated from zebrafish larvae with TRIzol® Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in accordance with the manufacturer's instructions. RNA purity (Abs 260/280 nm ~2.0) and concentration were determined by NanoDrop Lite (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and after treated with Deoxyribonuclease I – Amplification Grade (Sigma-Aldrich Inc., St Louis, Missouri, USA) to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, Madison, Wisconsin, USA) from 1 µg of the total RNA, following the manufacturer's instruction. Quantitative PCR was performed using SYBR® Green I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems, Foster City, California, USA). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers and showed in all cases one single peak. All real-time assays were carried out in quadruplicate and, in all cases, a reverse transcriptase negative control was included by substituting the templates for DNase/RNase-free distilled water in each PCR reaction. *EF1α* and *Rpl13α* were used as reference genes for normalization. The efficiency per sample was calculated using LinRegPCR 2018.0 Software (http://LinRegPCR.nl). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (Bustin et al., 2013; Pfaffl, 2001). The sequences of reverse and forward primers are in Table 1.

2.7. Evaluation of oxidative stress

Larvae were exposed to treatments for 24 h, using 20 larvae per treatment group. Larvae were stored at −80 °C in 500 µL of phosphate buffered saline solution (PBS, pH 7.3). After thawing, the samples were homogenized. The samples were centrifuged at 12000 rpm for 5 min at 4 °C and the supernatants were used for the assays (Leite et al., 2012). The activity of the enzyme catalase (CAT) was determined by UV spectrophotometry in accordance with the method described by Aebi (1984) and the enzyme glutathione (GSH) was determined by UV spectrophotometry in accordance with the method described by Shim et al. (2010). Proteins were determined according to Bradford (1976) by the Coomassie blue method, using bovine serum albumin as standard.

2.8. Statistical analysis

For the statistical analysis of the survival curve, it was used the Kaplan-Meier test. The results obtained in the other experiments were analyzed by one - way analysis of variance (ANOVA) followed by Tukey's test, considering $p < 0.05$ as statistical significance. Data are expressed as mean ± standard error.

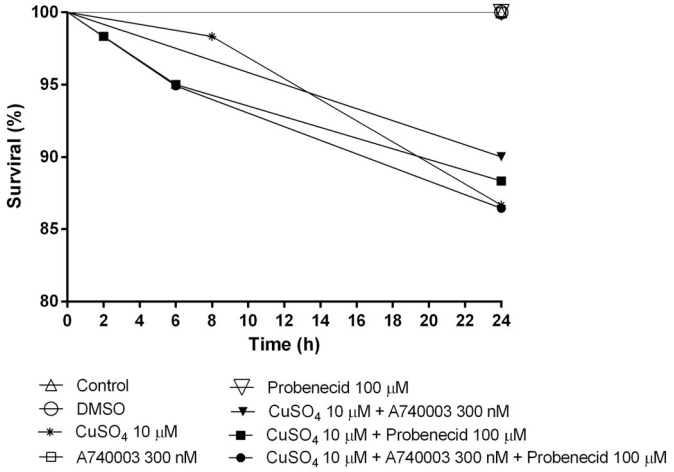

Fig. 2. Survival curve. Evaluation of the survival of the larvae treated with $CuSO_4$, A740003, probenecid, and associations. The animals were treated for 24 h and survival rates were determined at 2, 4, 8 and 24 h. The statistical analysis of the results was performed using the Kaplan–Meier method.

3. Results

In order to determine the ideal concentration to be used in the study, we performed a survival evaluation curve. After treatments, larvae survival rates were determined at 2, 4, 8 and 24 h. None treatments performed significantly decreased larvae survival when compared to control (100%) and DMSO (100%) (Fig. 2). The survival was 86.67% for 10 µM $CuSO_4$, 100% for 300 nM A740003, 100% for 100 µM probenecid, 90% for 10 µM $CuSO_4$ + 300 nM A740003, 88.33% for 10 µM $CuSO_4$ + 100 µM probenecid and 86.44% for 10 µM $CuSO_4$ + 300 nM A740003 + 100 µM probenecid.

The evaluation of locomotor activity 4 h post treatment showed a significant increase in the mean velocity of the larvae treated with $CuSO_4$ (0.0025 ± 0.0003) ($p < 0.05$), $CuSO_4$ + A740003 (0.0027 ± 0.0004) ($p < 0.05$), and $CuSO_4$ + A740003 + probenecid (0.0028 ± 0.0002) ($p < 0.01$; F [7110] = 4691), in relation to the control group (0.0015 ± 0.0002) (Fig. 3A). While for the mobile time, no significant difference was observed for any of the treatments tested during 4 h in relation to control. Larvae treated with $CuSO_4$ + A740003 (211.7 ± 21.66) increased significantly the mobile time in relation to A740003 single treatment (236 ± 14.13) ($p < 0.05$; F [7110] = 2.789) (Fig. 3B). During this same treatment period, the group treated with $CuSO_4$ + A740003 + probenecid (0.62 ± 0.08) presented an increase of the distance covered in relation to the control group (0.32 ± 0.06) ($p < 0.05$; F [7110] = 2.888) (Fig. 3C). There were no differences for any of the groups tested in turn angle parameter after 4 h treatment (F [7110] = 2.379) (Fig. 3D).

For animals treated for 24 h, a significant increase in mean velocity was observed in the animals treated with $CuSO_4$ (0.0038 ± 0.0006) ($p < 0.001$), $CuSO_4$ + probenecid (0.0033 ± 0.0003) ($p < 0.01$), $CuSO_4$ + A740003 + probenecid (0.0031 ± 0.0002) ($p < 0.05$), in relation to the control group (0.0017 ± 0.0001). It was also observed,

Table 1
Primer sequences for RT-qPCR experiments included in the study.

Gene	Forward primer	Reverse primer	Reference
EF1α	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′	Tang et al., 2007
Rpl13α	5′-TCTGGAGGACTGTAAGAGGTATGC-3′	5′-AGACGCACAATCTTGAGAGCAG-3′	Tang et al., 2007
PANX1α	5′-TTCGCTCAGGAAGTTTCTGTCGGT-3′	5′-ACTGCCACCAGCAGCAGGATATAA-3′	Bond et al., 2012
PANX1β	5′-TAAGTATAAAGGCGTGCGGCTGGA-3′	5′-ATACGCAGCCTGTCTCATCGTGAA-3′	Bond et al., 2012
P2X7	5′-TCCTGCAATGTGGCCAAAGCAG-3′	5′-TCTGGGTTTTGTCTGCCATTGTGC-3′	Cruz et al., 2013
IL1β	5′-ATGCTCATGGCGAACGTC-3′	5′-TGGTTTTAGTGTAAGACGGCACT-3′	Banerjee and Leptin, 2014
IL10	5′-TCACGTCATGAACGAGATCC-3′	5′-CCTCTTGCATTTCACCATATCC-3′	Faikoh et al., 2014

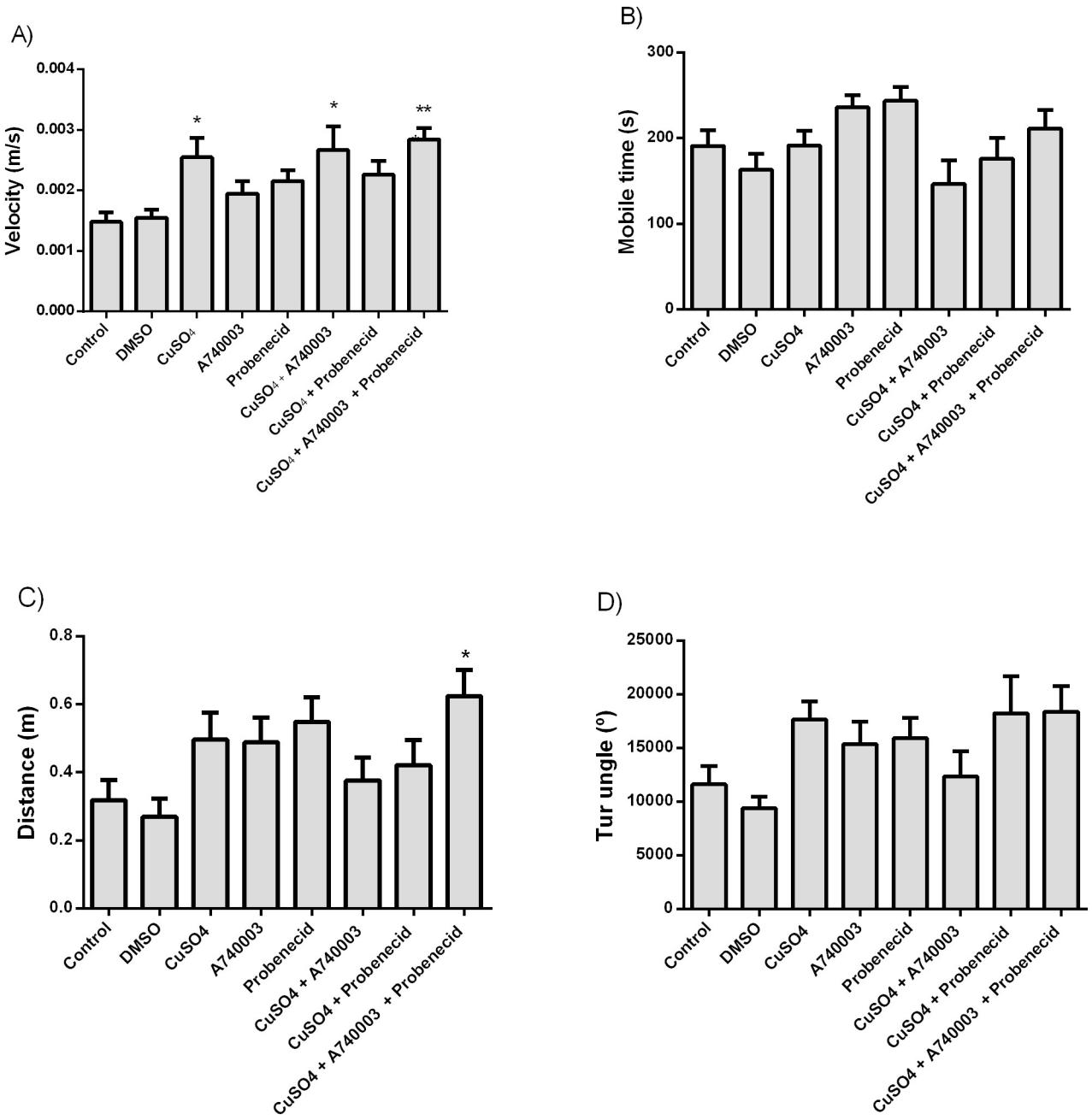

Fig. 3. Larvae locomotor activity after 4 h exposure. Effect of $CuSO_4$ 10 μM; A730003 300 nM; Probenecid 100 μM; $CuSO_4$ 10 μM + A730003 300 nM; $CuSO_4$ 10 μM + Probenecid 100 μM; $CuSO_4$ 10 μM + A730003 300 nM + Probenecid 100 μM. The parameters analyzed were velocity (A), mobile time (B), distance (C), turn angle (D). Values are expressed as mean ± SEM. Mean values significantly different from control group *P < 0.05, **P < 0.01, and ***P < 0.001. Mean values significantly different from $CuSO_4$ group $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001.

for this same parameter and time, that the groups that were not exposed to $CuSO_4$, A740003 (0.0017 ± 0.0001) ($p < 0.001$) and probenecid (0.0015 ± 0.0001) ($p < 0.001$; F [7103] = 10.74), presented a significant difference in the speed when compared to those exposed to $CuSO_4$ (Fig. 4A). For the mobile time evaluated after 24 h, the only group that presented a significant decrease in relation to the control (215.2 ± 21.99) was $CuSO_4$ (95.93 ± 22.63 $p < 0.05$). While the group probenecid + $CuSO_4$ (208.4 ± 21.29) showed a significant increase of the mobile time when compared to the group treated with only $CuSO_4$ ($p < 0.05$; F [7103] = 3.030) (Fig. 4B). Also, in this same period, there was observed a significant increase in the distance traveled in the group $CuSO_4$ + probenecid (0.74 ± 0.14) in relation to the $CuSO_4$ group (0.30 ± 0.06) ($p < 0.05$; F [7103] = 4.654) (Fig. 4C). We also analyzed the turn angle of the animals after 24 h exposure, in which a significant increase of this parameter was observed in the animals treated with $CuSO_4$ + probenecid (21,200 ± 3738). The increase was also observed both, in relation to the control group (9208 ± 1249) ($p < 0.05$), and in relation to the group treated with $CuSO_4$ (6212 ± 1189) ($p < 0.001$; F [7103] = 4.196) (Fig. 4D).

Since the blockage of PANX1 and P2X7R lead to changes in locomotor parameters after 24 h treatments, gene expression analysis was determined at this time point. We observed an increase in PANX1a channel gene expression (Fig. 5A) of the DMSO group (3.72 ± 1.16) in relation to the control (1.33 ± 0.37) ($p < 0.05$). The expression of this same gene was decreased in the animals treated with

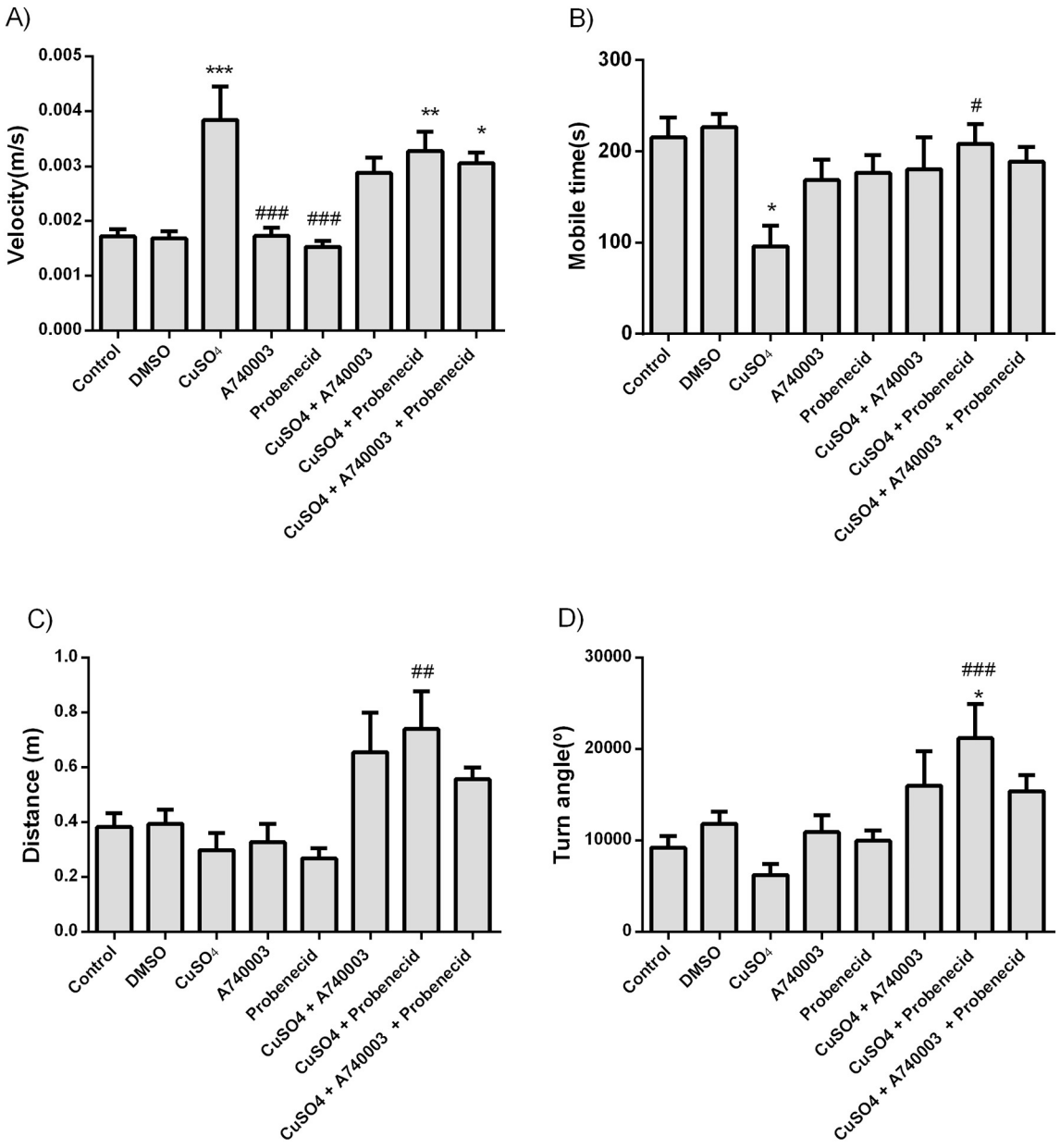

Fig. 4. Larvae locomotor activity after 24 h exposure. Effect of $CuSO_4$ 10 μM; A730003 300 nM; Probenecid 100 μM; $CuSO_4$ 10 μM + A730003 300 nM; $CuSO_4$ 10 μM + Probenecid 100 μM; $CuSO_4$ 10 μM + A730003 300 nM + Probenecid 100 μM. The parameters analyzed were velocity (A), mobile time (B), distance (C), turn angle (D). Values are expressed as mean ± SEM. Mean values significantly different from control group *P < 0.05, **P < 0.01, and ***P < 0.001. Mean values significantly different from $CuSO_4$ group $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001.

$CuSO_4$ + probenecid (0.94 ± 0.12) in relation to the DMSO group (3.72 ± 1.16) ($p < 0.01$; F [5,39] = 3.782). Although all groups expressed the PANX1b channel genes (F [5,40] = 1.277) (Fig. 5B) and the P2X7R (F [5,39] = 0.6740) (Fig. 5C), no significant differences in gene expression were observed. The analysis of interleukins IL-β (F [5,38] = 1.389) (Fig. 5D) and IL-10 (F [5,35] = 0.7619) (Fig. 5E), showed that all groups expressed interleukin genes, but treatments did not lead to significant differences the.

The evaluation of oxidative stress was performed through the determination of the enzymes glutathione reductase (GSH) (F [5,29] = 2.178) (Fig. 6A) and catalase activity (CAT) (F [5,24] = 0.4306) (Fig. 6B). There were no significant differences in the determination of the enzymes in any groups studied.

4. Discussion

In this study, we evaluated the effect of P2X7R-PANX-1 inhibition in locomotor activity and in inflammation induced by $CuSO_4$ in zebrafish larvae. Initially, a survival curve was performed and there was no significant decrease in survival of any of the treated groups, showing that the compounds did not cause lethal toxicity at the doses tested, even when associated, making them feasible for their use.

It was previously described that $CuSO_4$ can induce different profiles of inflammation according to the length of exposure: 4 h are representative of the initial phase of the inflammatory process, while 24 h of exposure have characteristics of a late stage of inflammation (Leite et al., 2013). Based on this previous study, we chose to use these two times to analyze the effect of the pharmacological treatments in larvae locomotor activity (velocity average, total distance, mobile time and turn angle).

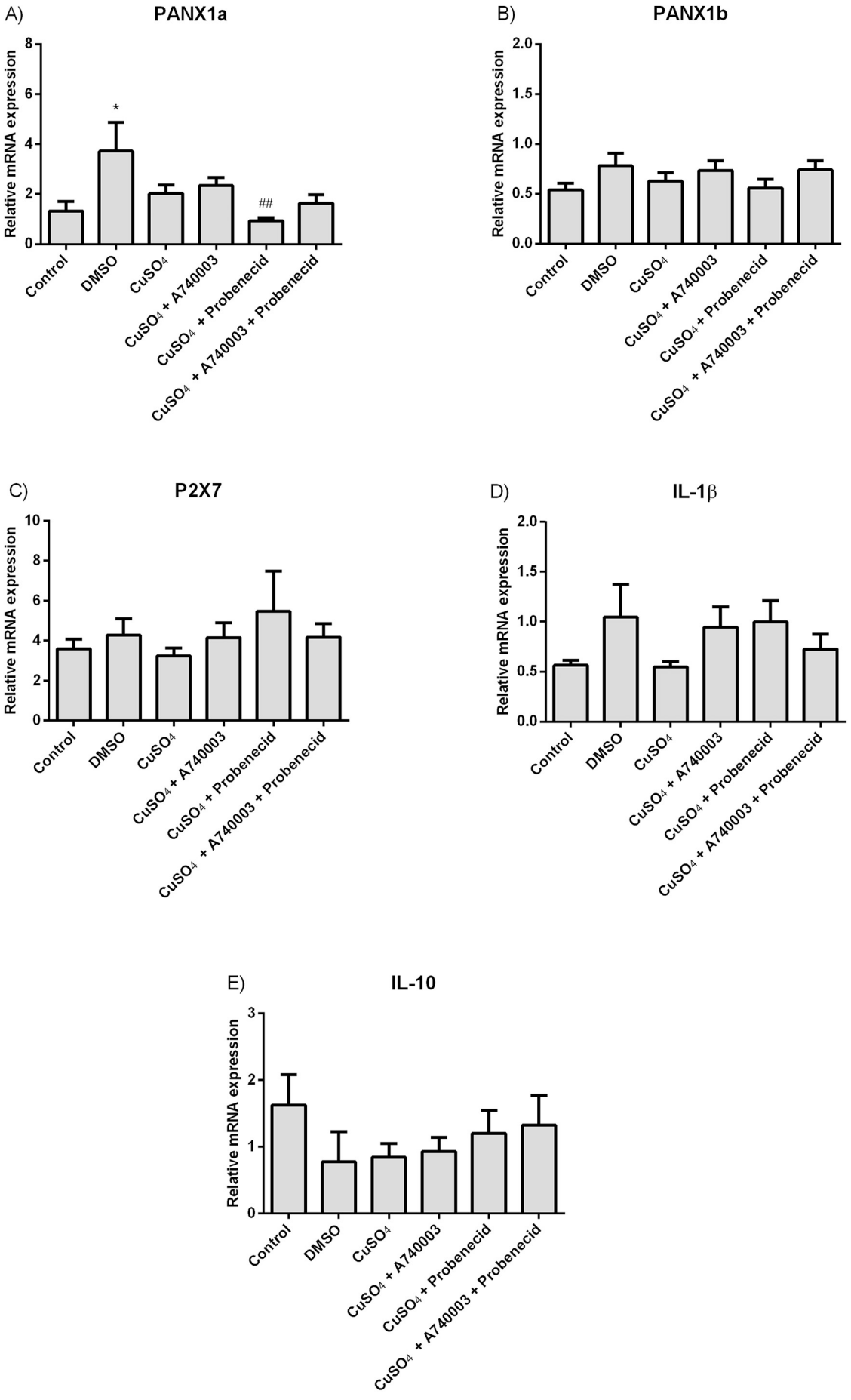

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Fig. 5. Gene expression of IL-1β (A), IL-10 (B), PANX1a (C), PANX1b (D) e P2X7R (E) after 24 h exposure. Effect of $CuSO_4$ 10 μM; $CuSO_4$ 10 μM + A730003 300 nM; $CuSO_4$ 10 μM + Probenecid 100 μM; $CuSO_4$ 10 μM + A730003 300 nM + Probenecid 100 μM. It was used a pool of 20 larvae per group (n = 5). Values are expressed as mean ± SEM. Mean values significantly different from control group *P < 0.05, **P < 0.01, and ***P < 0.001. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. Mean values significantly different from DMSO group $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001.

Here, we observed that animals treated during 4 h with $CuSO_4$ showed little or no difference in locomotor activity, whereas those treated for 24 h demonstrated significant changes, suggesting that the effects of copper-induced inflammation in larvae locomotion occur after a longer exposure time. It is interesting to note that only the groups treated with $CuSO_4$, alone or in combination, showed differences in locomotion, indicating that this effect is caused by the inflammatory agent. In this study, we used parameters widely described in the evaluation of larvae locomotor activity, such as distance traveled, velocity and turn angle (Colwill and Creton, 2011; F. Li et al., 2018). According to our results, the mobile time was the main parameter impairing larvae locomotor activity after the pharmacological treatments. In fact, the group exposed to $CuSO_4$ presented a decrease in mobile time and an increase in speed in 24 h. The decrease of the mobile time is indicative of hypoactivity and the increase of velocity an indicative of escape, both described as behavioral characteristics of zebrafish larvae with the presence of disease or pain (Kalueff et al., 2013). These results confirm our proposed inflammatory model since $CuSO_4$ was intended to induce inflammation.

It is already known that ATP is involved in the inflammatory process, presenting pro-inflammatory characteristics through the activation of the P2X7 receptor (Burnstock and Knight, 2018). In this study, proposed to block P2X7R and/or the PANX-1 channel in order to investigate their participation in copper-induced inflammation. Interestingly, zebrafish exposed only to $CuSO_4$ presented a decreased in mobile time, and the inhibitor of -PANX-1 channel reverted the effect of $CuSO_4$, indicating that this pathway may influence the locomotor activity of the larvae. Animals treated with the PANX-1 channel inhibitor also presented significant changes, in relation to the $CuSO_4$ group, in the distance and the turn angle. A study noted in the mouse model that PANX-1 channels may contribute to inflammatory response and neurobehavioral changes (Wu et al., 2017).

In an interesting way, the mean velocity parameter presented similar characteristics in the two times of treatment, in which an increase of the speed was observed in the groups treated with $CuSO_4$ and the treatments were not able to revert this effect. We could infer that, probably, this effect is being triggered by another pathway which justifies the non-reversion of this effect by blocking P2X7R and/or PANX-1. Changes in locomotor parameters may be related to more than one effect, although the increased velocity is indicative of pain and is also described as an anxiety parameter (Kalueff et al., 2013). Another study also using $CuSO_4$ to induce inflammation in zebrafish larvae showed that exposure to this inflammatory agent had similar anxiogenic characteristics to those observed with caffeine (Cruz et al., 2017).

For the analysis of the gene expression and of the evolution of oxidative stress, we chose the time of 24 h of treatment, time in which the alterations in locomotor activity presented the greater difference between the treated groups. Although we know the involvement of P2X7R and PANX-1 in inflammation, and it has been already shown the role of the purinergic system in this inflammatory model (Cruz et al., 2017; Leite et al., 2013), this is the first time that P2X7 and PANX-1 are evaluated in this model of inflammation. Unlike humans, zebrafish presents two variations of the PANX-1 channel, called PANX1a and PANX1b, which have different distribution profiles, and PANX1a is closest to the mammalian expression profile. In addition to the different distribution profiles, they also present some distinct physiological properties (Bond et al., 2012). Although the genes encoding the P2X7R and the PANX-1 channel have been expressed, evidencing the presence of these targets in zebrafish, pharmacological modulation did induce significant differences in the expression of these genes. However, we observed that treatment with DMSO, used for dilution of probenecid and A740003, increased the expression of the PANX1a gene. We also observed that larvae treated with $CuSO_4$ plus probenecid diminished the expression of the PANX-1 channel. Although DMSO has low toxicity and is routinely employed in the dilution of drugs for biological assays. It is worth to mention that there are reports of various effects in trials conducted with zebrafish, mainly behavioral effects, which were not seen here (Chen et al., 2011; Hallare et al., 2006). In this study, DMSO caused an increase the expression of the PANX1a gene. There is no evidence that the DMSO treatment is causing a toxic effect or inducing

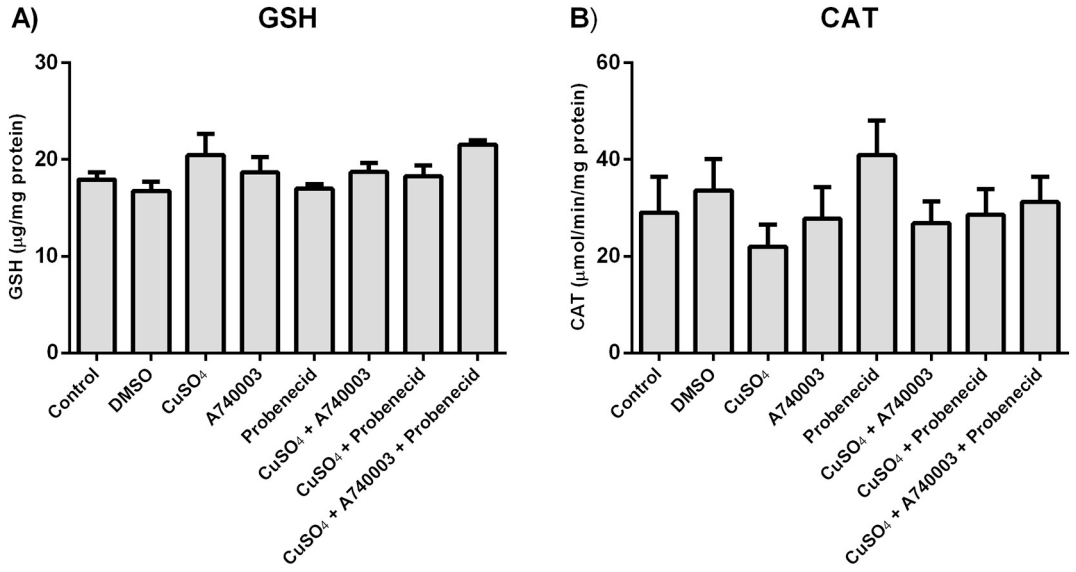

Fig. 6. Evaluation of oxidative stress in zebrafish larvae after 24 h exposure. Effect of $CuSO_4$ 10 μM; $CuSO_4$ 10 μM + A730003 300 nM; $CuSO_4$ 10 μM + Probenecid 100 μM; $CuSO_4$ 10 μM + A730003 300 nM + Probenecid 100 μM on GSH activity (A) and CAT activity (B). Mean values significantly different from control group *P < 0.05, **P < 0.01, and ***P < 0.001. Mean values significantly different from $CuSO_4$ group $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001.

inflammation in our findings, since the treatment did not trigger changes in the survival curve, in oxidative stress determinations, and in the inflammatory markers gene expression. We also evaluated the effect of $CuSO_4$ and treatments on IL-1β (pro-inflammatory) and IL-10 (anti-inflammatory) gene expression after 24 h of exposure. It was not possible to observe significant differences between the treated groups; in fact, the IL-10 anti-inflammatory cytokine did not present difference in the expression of the animals exposed to $CuSO_4$ alone in other studies (Leite et al., 2013), the decrease was only observed when associated with caffeine, which presented characteristics pro-inflammatory (Cruz et al., 2017). It is known that P2X7R and/or PANX1 channel are directly related to the release of IL-1β (Pelegrin and Surprenant, 2006), surprisingly we observed no difference in IL-1β expression in the groups in which P2X7 receptor inhibition. However, other studies have shown that despite the involvement of P2X7-PANX1 in the activation of NLRP3 inflammasome and consequently in the release of IL-1β, the inflammasome can be activated by other independent pathways (Qu et al., 2011).

It is known that IL-1β release process includes two main steps: the first dependent on the activation of NF-κB which will induce the expression of premature IL-1β forms, and the second involves the formation of the inflammasome which, with caspase-1, is responsible for the cleavage of the premature IL-1β forms, producing the fully active cytokines that will be released (Crespo Yanguas et al., 2017). We could suggest that the blockage of P2X7R and/or the PANX1 channel used in this study leads to the inhibition only the second stage of the chronic stage of inflammation.

We evaluated the enzymatic activity of two important enzymes with antioxidant activity involved in this process: catalase (CAT) and glutathione reductase (GSH). We observed no difference in $CuSO_4$-treated animals, we know that $CuSO_4$ causes oxidative stress in the larvae and that this effect can be observed shortly after 40 min of exposure (Olivari et al., 2008). It is worth to mention that we observed a reduction in catalase activity when the animals were treated with $CuSO_4$, but this result did not achieve significance. Besides, there are other oxidative stress markers that could be evaluated in zebrafish, such as TBARS determination (Sehonova et al., 2019), which could contribute to the clarification of the oxidative stress triggered by $CuSO_4$. We performed our evaluations after 24 h of exposure to $CuSO_4$, it is possible that changes in these enzymes can be observed in the first hours of exposure to $CuSO_4$. Even no significant differences were observed in gene expression and in oxidative stress, the results in the behavioral tests showed that $CuSO_4$ is capable of causing considerable changes in the behavioral pattern of the larvae. In fact, a previous study using different organisms reports the influence of $CuSO_4$ on neurotransmission systems, such as dopaminergic (Bonilla-Ramirez et al., 2011; Opazo et al., 2014). Also, it has already been observed that the inhibition of PANX-1 may interfere with NMDA-induced ion currents (S. Li et al., 2018; Weilinger et al., 2012). Therefore, we cannot rule out the hypothesis that some of the effects caused by inhibition of the PANX-1 channel are related to the modulation of crosstalk between PANX-1 and NMDA receptor channels.

In conclusion, data presented herein show, for the first time, the relevance of the P2X7-PANX-1 blockage in the late stage of inflammation induced by $CuSO_4$. The mechanisms by which these alterations are elicited still need to be better investigated.

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