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Original Article

Effect of Anakinra (Kineret®) treatment on acrylamide-induced neurotoxicity in mice

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ABSTRACT — Recent studies demonstrated that upregulation of proinflammatory cytokines were associated with neurotoxicity of acrylamide, which is used widely in industries and generated in food cooked at high temperature. The interleukin-1 (IL-1) is one of cytokines that play an important role in immune response. Anakinra is an IL-1 receptor antagonist used as anti-inflammatory medicine against inflammatory diseases such as juvenile idiopathic arthritis. In this study, ten-week old wild type male mice were allocated into 6 groups. Group 1 to 3 daily received subcutaneous injection with vehicle and oral exposure to ACR in drinking water at 0, 150 or 300 ppm for 28 days, and group 4-6 daily received injection with Anakinra and oral exposure to ACR in drinking water at 0, 150 or 300 ppm for 28 days. The landing foot spread (LFS) test was carried out to assess the motor function, and immunohistochemistry was carried out for quantification of noradrenergic axons and microglia activation. The results of LFS did not show significant effect of Anakinra treatment on ACR-induced increase in landing foot spread in mice. The body weight was dose-dependently decreased by ACR exposure only in the groups treated with Anakinra. The IHC staining for microglia and noradrenergic axon density does not show any significant effect of treatment with Anakinra or exposure to ACR. The study demonstrated that daily treatment with Anakinra at 25 mg/kg body weight does not ameliorate ACR-induced neurotoxicity in mice, while potentiates ACR-induced loss of body weight.

Key words: Anakinra, Acrylamide, Neurotoxicity, Noradrenergic axons, Microglia, Neuroinflammation

INTRODUCTION

Acrylamide is an environmental electrophile that is extensively utilized in various industries (Ruenz *et al*., 2016). Additionally, acrylamide can be formed unintentionally in certain foods during high-temperature cooking processes, through a chemical reaction known as the Maillard reaction (Tareke *et al*., 2000, 2002). Acrylamide is widely recognized as a neurotoxicant and has been shown to induce neuropathies and encephalopathies in both humans and experimental animals. Its neurotoxic effects have been the focus of numerous studies (Erkekoğlu and Baydar, 2010; LoPachin, 2004, 2002a, 2002b; Spencer and Schaumburg, 1974).

Indeed, emerging evidence suggests that neuroinflammation may be an underlying mechanism of acrylamide-induced neurotoxicity in mice (Ekuban *et al.,* 2021; Zong *et al.,* 2019). Studies have demonstrated that acrylamide-induced neurotoxicity in rats and BV-2 microglia cell lines is associated with an upregulation of pro-inflammatory cytokine genes (Zong *et al*., 2019). This suggests that the inflammatory response mediated by pro-inflam-

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matory cytokines may contribute to the neurotoxic effects of acrylamide.

According to the study by Ekuban and his colleagues (Ekuban *et al*., 2021), acrylamide-induced sensorimotor dysfunction and degeneration of monoaminergic axons in the prefrontal cortex were observed in Nrf2-null mice compared to wild-type mice. This degeneration was accompanied by microglia activation and an upregulation of pro-inflammatory cytokine genes. The absence of Nrf2, a transcription factor involved in cellular defense mechanisms and downregulation of proinflammatory cytokines, may have contributed to the increased susceptibility to acrylamide-induced neurotoxicity through activation of inflammatory response.

Kineret® (Anakinra) is a potent and highly selective competitive antagonist of interleukin-1 (IL-1). It is known to block all actions of IL-1 and is induced by tissue injury (Loddick *et al*., 1997; Pinteaux *et al*., 2006). In rodent studies, IL-1 receptor antagonist (IL-1RA) has shown significant inhibition of neuronal injury caused by various conditions such as focal or global cerebral ischemia, excitotoxicity, traumatic brain injury, and seizures (Relton and Rothwell, 1992; Touzani *et al*., 1999).

Interleukin-1 (IL-1) exists in two forms, IL-1a and IL-1b, which are separate ligands but exert similar biological effects. Both IL-1a and IL-1b bind to the membrane-bound IL-1 receptor I (IL-1R1). This binding leads to the recruitment of two IL-1 receptor accessory proteins, IL-1RAcP (IL-1 receptor accessory protein) and IL-1RAcPb, to form a receptor complex. This complex enables intracellular signaling and the induction of downstream inflammatory mediators. The activation of IL-1 signaling pathway plays a crucial role in the initiation and regulation of inflammatory responses in various physiological and pathological conditions.

IL-1a and IL-1b are synthesized by various cell types in both the peripheral and central immune systems, including astrocytes, microglia, neutrophils, lymphocytes, and monocytes (Allan *et al*., 2005) . The mechanisms of action of IL-1 are complex and not fully understood, but they involve several processes. Firstly, IL-1 can induce the release of neurotoxic substances, such as matrix metalloproteinase-9 (MMP-9), from astrocytes, which can contribute to neuronal damage (Thornton *et al*., 2008). Secondly, IL-1 can activate brain endothelium, leading to increased permeability of the blood-brain barrier and recruitment of immune cells into the brain (Konsman *et al*., 2004). Thirdly, IL-1 can stimulate and facilitate the invasion of leukocytes into the brain, which is important for the inflammatory response (Bernardes-Silva *et al*., 2001; McColl *et al*., 2007). Lastly, IL-1 can also have actions on the extracellular matrix, potentially affecting tissue remodeling and repair processes (McColl *et al*., 2008; Summers *et al*., 2009). The naturally occurring IL-1 receptor antagonist (IL-1RA) competitively binds to IL-1R1 and effectively blocks the actions of both IL-1a and IL-1b (Hannum *et al*., 1990). This property makes IL-1RA a potential therapeutic option for various conditions.

In this study, we test the hypothesis of inhibition of IL-1 using Anakinra could have a protective effect against acrylamide induced neurotoxicity in mice brain.

MATERIALS AND METHODS

Chemicals and preparation

Acrylamide (lot #A9099, purity > 99%) was purchased from Sigma Aldrich (St. Louis, MO, USA), and Anakinra (Kineret®) was purchased from SObi. Acrylamide was freshly prepared at the beginning of each week by dissolving in a G-10 ion exchange cartridge (Organo Co., Tokyo, Japan) filtered drinking water, stored at 4 degrees Celsius and administered every day in autoclaved bottles. Anakinra (Kineret®) is used in (100 mg/ 0.67 mL), briefly 20 mg from (Kineret®) was withdrawn from the syringe and diluted with saline. Vehicle of (Kineret®) was prepared according to the instruction in (Kineret®) prescription which consists of (Each prefilled glass syringe contains: 0.67 mL (100 mg) of anakinra in a solution (pH 6.5) containing anhydrous citric acid (1.29 mg), disodium EDTA (0.12 mg), polysorbate 80 (0.70 mg), and sodium chloride (5.48 mg) in Water for Injection, USP).

Animal husbandry and experimental design

Ten-week-old male mice were used. Twenty four male specific-pathogen free C57BL/6msSIc mice were purchased from (SLC Japan, Inc., Tokyo, Japan) at 8-weeks of age and allowed to acclimatize for two week before the start of study. Mice were initially housed in separate cages of six each and had access to filtered drinking water and normal chow diet (Charles River Formular-1; 5LR1) ad libitum. They were housed in a controlled environment of temperature (23–25°C), humidity (57–60%) and light (lights on at 0800 hr and off at 2000 hr). After the second week of acclimatization the mouse was weighed and then assigned at random to one of six groups, each consisting of 4 mice, which were allocated into acrylamide only (0, 150 or 300 ppm) or acrylamide plus Anakinra (Kineret®) co-exposure groups. Groups 1 to 3 were exposed to acrylamide at 0, 150, 300 ppm and co-treated with vehicle as mentioned below, respectively, while groups 4, 5 and 6 were exposed to acrylamide

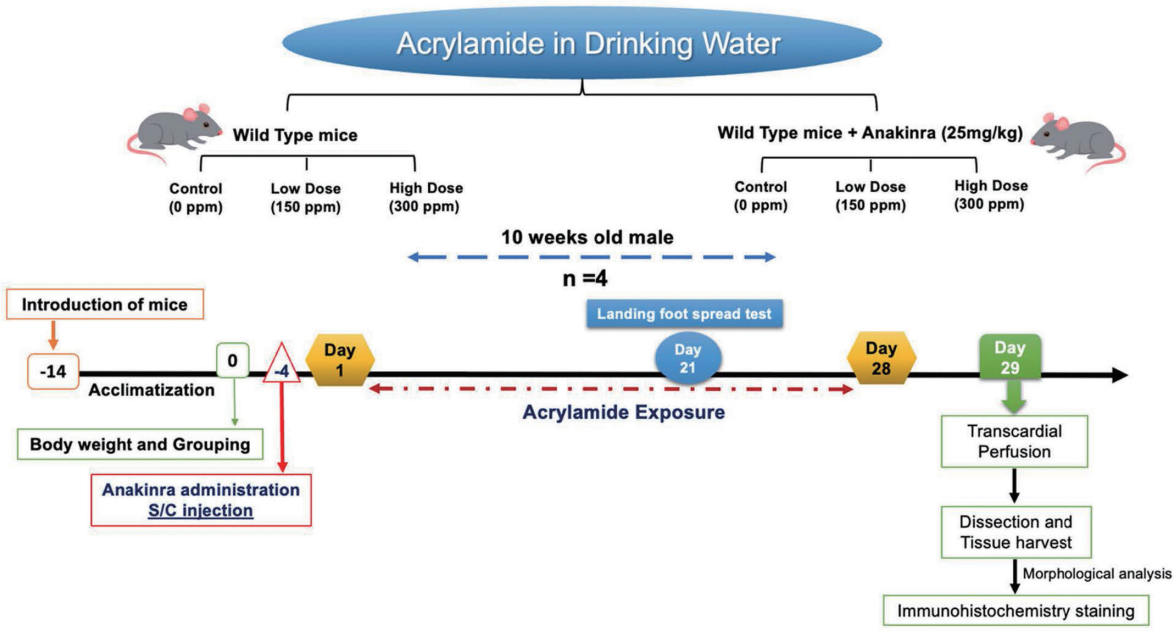

Fig. 1. Schematic illustration of the acrylamide- Anakinra study. All the mice ingested water containing acrylamide for four weeks, the landing foot spread test was done at the day 21 to assess the motor function then, morphological analysis was done using immunohistochemical staining of noradrenergic axons and microglia.

at 0, 150, 300 ppm respectively, co-treated with Anakinra (Kineret®) at 25 mg/kg body weight (modified from, Greenhalgh *et al*., 2010). Acrylamide was added to the drinking water whereas Anakinra (Kineret®) was administered through sub-cutaneous injections. Mice of groups 1 to 3 also received subcutaneous injections of vehicle (0.67 mL (100 mg) of Anakinra in a solution (pH 6.5) containing anhydrous citric acid (1.29 mg), disodium EDTA (0.12 mg), polysorbate 80 (0.70 mg), and sodium chloride (5.48 mg)). Mice of each group (n = 4 each) were housed in four per cage and treated with the compounds every day for four weeks (Fig. 1).

In the present study, Anakinra was used according to previous animal experiments at dose 25 mg/kg/day for 45 days in mdx mice model of Duchenne muscular dystrophy (Benny Klimek *et al.,* 2016), at 25 mg/kg twice daily for five days in mice model of seizures (Taraschenko *et al.,* 2021) and the same dose in the ApoE$^{-/-}$ mice model of arthrosclerosis (Ku *et al*., 2022).

The dose of 100 mg per person was used for treatment of human cases in refractory cerebral autoinflammatory–autoimmune diseases (Jang *et al.,* 2022). The dose at 2 mg/kg daily for 2 years was used in the intracranial Erdheim-Chester disease (ECD).

For acrylamide, 300 ppm was used as the highest exposure level based on the findings of previous studies (Gilbert and Maurissen, 1982) and the fact that it matches the levels of human exposure to acrylamide at 400 ppm from a polluted drinking well water (Igisu *et al*., 1975; Morimoto, 1975). Moreover, in a series of preliminary studies, 300 ppm of acrylamide induced signs of neurotoxicity in experimental mice, without causing mortality. Following the 4-week exposure, mice were fixed by transcranial perfusion under deep anesthesia for the purposes of morphological analysis.

The protocol and experimental design of the present study were approved by the animal experiment committee of the Tokyo University of Science and strictly followed the guidelines of Tokyo University of Science on animal experiments in accordance with the Japanese act on welfare and management of animals.

Amount of acrylamide and drinking water uptake

The body weight of all mice as well as the amount of water consumption were measured and recorded every day between 10:00 and 11:00 am. The amount of water consumption across the groups was also measured. The mean of the drinking water over 4 weeks period of acryla-

Table 1. Average drinking water consumed in acrylamide-Anakinra treated mice.

Test parameters	Treatment	Concentration of Acrylamide (ppm)		
		0	150	300
Acrylamide drinking water amount/body weight (mL/kg.bw)/ exposure group	Anakinra (-)	0.725 ± 0.51	0.671 ± 0.046	0.461 ± 0.041
	Anakinra (+)	0.916 ± 0.089	0.52 ± 0.03	0.515 ± 0.035
Acrylamide drinking water amount/body weight (mL/kg.bw)/ mouse	Anakinra (-)	0.181 ± 0.013	0.168 ± 0.012	0.115 ± 0.01
	Anakinra (+)	0.229 ± 0.022	0.13 ± 0.008	0.129 ± 0.009

Abbreviation: ACR, acrylamide. Data are mean ± SD. *p < 0.05, ** p < 0.01, compared with the control by Dunnett's multiple comparison following ANOVA for the body weight (n = 4).

mide exposure is shown in Table 1. The water consumption does not show a significant effect in both Anakinra (-) and Anakinra (+) for all tested groups.

Landing foot spread test

The landing foot spread test was performed following the protocol of the functional observatory battery testing for the effects of drugs and other chemicals on the nervous system recommended by the United States Environmental Protection Agency (USEPA) and as described previously (Edwards and Parker, 1977; Gilbert and Maurissen, 1982). Briefly, mice were dropped from a height of 15 cm after applying a foot dye ink to the soles of the hindlimb. The distance of hindlimb spread upon landing was recorded as the hindlimb splay length. The test was carried out three times and the mean landing foot spread value was reported.

Perfusion and morphological assessment

At 24 hr after the last exposure of acrylamide, mice (n = 4/group) were deeply anesthetized with intraperitoneal injection of sodium pentobarbitone (50 mg/kg). Upon confirmation of loss of sensation, the animals were transcardially perfused through the ascending aorta with 4% paraformaldehyde in phosphate buffer (4% PFA). The perfused mice were wrapped in aluminum foil and kept on ice for a period of 1 hr to increase the penetrative effect of paraformaldehyde particularly through the brain tissues. The brain was dissected out of the skull carefully and fixed for additional 24 hr at 4°C. After this, the brain was divided into three parts by cutting coronally at the anterior margin of the cerebellum and the optic chiasm and then placed in a series of 10, 20 and 30% sucrose solutions over changing intervals of 24 hr each. Brain tissues (PFC area) were then embedded in optimum cutting temperature (OCT) medium with the use of plastic Tissue Tek cryomolds (SFJ 4566, Sakura, Japan) and then stored at -80°C.

Region of interest for assessment of CNS-neurotoxic effect

Noradrenergic and Iba1-immunopositive microglia were quantified in the medial prefrontal cortex. The prefrontal cortex (PFC) is an important region of the brain known to be involved in the planning of complex cognitive processing, personality expression, decision making as well as social or emotional behaviors in humans. The PFC is innervated with many afferent and efferent axons. Dysfunction or degeneration of axon terminals in the PFC has been implicated in several disorders such as depression, schizophrenia among others in humans (Ishikawa *et al*., 2007).

Cryosection of brain tissues

Brain tissues embedded in O.C.T. medium was serially sectioned in the coronal plane on a freezing microtome (Leica CM3050S, Leica Microsystems, Wetzlar, Germany) at 40 µm thickness from bregma 3.20 to 1.54 mm, according to Paxinos and Franklin (Paxinos and Franklin, 2004), which is representative of the full extent of mouse medial-prefrontal cortex. The tissue sections were placed on positively charged slides (Matsunami MAS superfrost slides, Matsunami Glass Ind., Osaka, Japan) and allowed to air-dry at room temperature for about 1 hr, after which they were stored at -80°C pending immunostaining.

Immunohistochemical examination

Noradrenergic axon and Microglial staining

The frozen OCT-embedded tissues were cryosectioned at 40 µm thickness using a cryostat (Leica CM3050S, Leica Microsystems, Wetzlar, Germany). The frozen sections were mounted on a Matsunami MAS superfrost glass slides (Matsunami Glass Ind., Osaka, Japan) and allowed to dry at room temperature for about 1 hr. Briefly, the air-dried sections were rinsed in Tris buffered saline (TBS; 50 mM Tris, 0.15 mM NaCl, pH 7.5-7.8) and transferred into an antigen retrieval solution containing 10 mM sodium citrate buffer (pH 8.5) that had

been pre-heated and maintained in a water bath at 80°C for 30 min. After the incubation, the sections were cooled to room temperature together with the buffer solution and washed in Tris-buffered Saline with 0.01% Tween-20 (TBST). Endogenous peroxidase activity was blocked for 20 min by incubating the sections with peroxidase blocking reagent (20 mL of 0.2 M phosphate buffer, 8 mL methanol, 80 µL Triton-X 100, 2 mL 30% hydrogen peroxide; diluting to 40 mL with distilled water). After triple washing in TBST, non-specific protein binding was blocked at 4°C overnight using protein blocking reagent [1% bovine serum albumin (BSA; Sigma Aldrich), 2.5% normal horse serum (NHS; Vector Laboratories, Burlingame, CA, USA), 0.3M glycine (Wako) and 0.1% Tween-20 (Wako)]. This was followed by brief incubation at 37°C for 30 min followed by rinsing three times in TBST. Endogenous interferences of Avidin-Biotin were blocked by incubating the sections in avidin/biotin blocking reagent (Sp-2001; Vector Laboratories), as described by the manufacturer. The sections were then incubated for 2 hr at 37°C with mouse anti-noradrenaline transporter antibody (NAT; 1:1000, #ab211463, Abcam, Japan) or incubated for 1 hr at room temperature with rabbit anti-Iba1 microglia (1: 500; Wako, Japan). Following incubation with the primary antibody, the sections were washed three times and then incubated for 1 hr with horse anti-mouse biotinylated secondary antibody (BA-2000; Vector Laboratories) for noradrenalin transport axon or incubated for 1 hr with horse anti-rabbit biotinylated secondary antibody (BP-100; Vector Laboratories) for microglia staining and further washed three times in TBST. Finally, the sections were stained with the avidin-biotin peroxidase complex (Elite ABC reagent, Vector Laboratories) and visualized by reacting with diaminobenzidine peroxidase substrate as the chromogen (ImmPACT DAB (Brown) peroxidase substrate SK-4105, Vector Laboratories), the DAB peroxidase reaction was stopped and rinsed with water, followed with three times in TBS. The sections were wiped off any liquid, allowed to air-dry and mounted with an aqueous mounting medium (VectaMount Mounting Medium, H-5501, Vector Laboratories).

Morphometric analysis of noradrenergic axons

Uncompressed photomicrographs of the stained prefrontal cortex regions were taken with a Leica FlexCam C1 digital camera-assisted microscope (BX 50, Olympus, Tokyo) (dm-PFC and vm-PFC), using the whole area with the vessel analysis plugin in the ImageJ software (Schneider *et al*., 2012). We determined the NA axon density, vascular density of NA transport, and the microglia process length and microglia area which represented the ratio of the vessel area relative to the total area multiplied by 100%. These studies were conducted in 4 mice and 2 sections from each were used for analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA) or JMP (version 14, SAS Institute, Cary, NC, USA). Data are expressed as mean ± standard deviation (SD), as indicated. Differences among groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Single regression analysis was carried out on the dose of acrylamide in each mouse treatment groups (acrylamide only and acrylamide+ Anakinra group) to determine the effects of Anakinra and trend with the dose of acrylamide using dummy variables for treatment. Multiple regression analysis with dummy variable (0: without Anakinra and 1: with Anakinra) was carried out to determine the interaction between the dose of acrylamide and Anakinra treatment. A model of multiple regression analysis without interaction was used to test the effects of the dose of acrylamide and Anakinra when their interaction was not significant. A probability of $p < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

Body weight

Dunnite multiple comparison following ANOVA showed that exposure to acrylamide at 150 and 300 ppm significantly decreased body weight only in Anakinra-treated mice (Table 2). Surprisingly, during animal handling at the time of the experiment, we noticed that groups of mice that take Anakinra were very healthy and showed aggressiveness from the 3rd week of exposure until the end of the experiment compared with the ACR only groups.

Functional changes (Landing foot spread test)

Dunnett's multiple comparison following ANOVA showed that exposure to ACR significantly and dose-dependently increased the hindlimb splay at 300 ppm either in Anakinra-treated or vehicle-treated mice (Fig. 2), but t-test did not show significant difference between mice treated with vehicle and mice treated with Anakinra when exposed to ACR either at 150 ppm or 300 ppm. Multiple regression analysis showed no signif-

Table 2. ANOVA test analysis of acrylamide effect and Anakinra on the body weight in wild type mice.

Test parameters	Treatment	Concentration of Acrylamide(ppm)			Simple regressioncoefficient of ACR(p value)	Multiple regression (p value)		
		0	150	300		Interaction of ACR and Anakinera	Regression coefficient of ACR	Regression coefficient of Anakinera treatment
Body Weight (g)	Anakinra (-)	26.90 ± 1.09	27.42 ± 1.90	25.94 ± 1.44	-0.0032 ± 0.3942	-0.0053 ± 0.2472	-0.0032 ± 0.3249	-0.6275 ± 0.2679
	Anakinra (+)	27.84 ± 1.19	25.27 ± 0.84**	25.27 ± 0.84**	-0.0085 ± 0.0097*			

Abbreviation: ACR, acrylamide. Data are mean ± SD. *p < 0.05, ** p < 0.01, compared with the control by Dunnett's multiple comparison following ANOVA for the body weight (n = 4).

Table 3. ANOVA and regression analysis summary for the effects of Anakinera and acrylamide exposure level on activation of microglia in dorso-medial prefrontal cortex (d-mPFC) and ventro-medial prefrontal cortex (v-mPFC).

Test parameters	Region	Treatment	Concentration of acrylamide (ppm)			Simple regressioncoefficient of ACR(p value)	Multiple regression (p value)		
			0	150	300		Interaction of ACR and Anakinra	Regression coefficient of ACR	Regression coefficient of Anakinra treatment
Microglia Area	d-mPFC	Anakinera (-)	6750.49 ± 2352.9	5313.4 ± 224.6	5714.5 ± 3285.9	-0.0161 ± 0.53	4.69 ± 0.65	-3.45 ± 0.64	-1347.6 ± 0.30
		Anakinera (+)	6835.6 ± 400.1	7778.5 ± 4954.4	7207.4 ± 2527.9	0.0015 ± 0.89			
	v-mPFC	Anakinera (-)	7324 ± 4241.4	5392.8 ± 297.7	7303.3 ± 881.7	0.0012 ± 0.95	4.31 ± 0.68	0.23 ± 0.97	953.2 ± 0.46
		Anakinera (+)	6735.7 ± 4241.4	7953.4 ± 4663.2	8100.5 ± 1979.2	0.0047 ± 0.64			
Microglia Process Length	d-mPFC	Anakinera (-)	235.3 ± 28.7	115 ± 2.20	145.7 ± 17.6	1.16 ± 0.53	-0.026 ± 0.76	0.034 ± 0.56	-2.04 ± 0.85
		Anakinera (+)	128.5 ± 30.7	134.5 ± 36.7	129.02 ± 23.3	0.174 ± 0.90			
	v-mPFC	Anakinera (-)	124.4 ± 39.6	121.1 ± 7.5	144.8 ± 12.8	0.214 ± 0.89	0.042 ± 0.65	0.008 ± 0.90	-0.664 ± 0.95
		Anakinera (+)	124.3 ± 30.3	142.4 ± 34.1	139.5 ± 27.42	0.96 ± 0.48			

Abbreviation: ACR, acrylamide. Data are mean ± SD. *p < 0.05, compared with the control by Dunnett's multiple comparison following ANOVA for the body weight (n = 4).

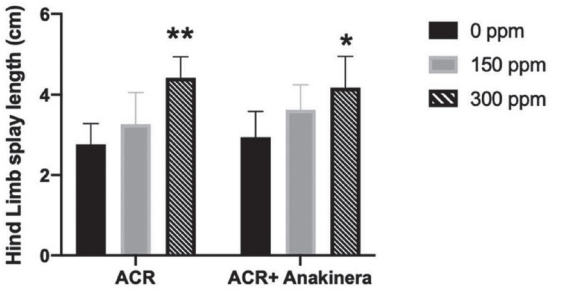

Fig. 2. Landing foot spread (LFS) test results of acrylamide exposed mice (0, 150, 300 ppm ACR) and (0, 150, 300 ppm ACR+ 25 mg/kg Anakinra). Dunnett's multiple comparison following ANOVA test show that the LFS significant increase ($P < 0.01$) in 300 ppm ACR and significant increase ($P < 0.05$) in 300 ppm ACR+ Anakinra. Mean ± SD. $*P < 0.05$, compared to the corresponding control by Dunnett's multiple comparison following ANOVA. (n = 4).

icant interaction between acrylamide and Anakinra treatment, suggesting no effect of Anakinra treatment on the intensity of ACR-induced increase in landing foot spread.

Morphological changes

NAT-immunoreactive (Noradrenergic axons)

Dunnett's multiple comparison following ANOVA showed that exposure to ACR did not change the density of noradrenergic axons in the dm-PFC and vm-PFC at 300 and 150 ppm in either vehicle- or Anakinra-treated mice (Figs. 3 and 4).

Iba1-immunoreactive microglia

Dunnett's multiple comparison following ANOVA showed that exposure to ACR did not change the area or process length of Iba1-positive microglia either in vehicle- or Anakinra-treated mice (Fig. 5, Table 3). Multiple regression analysis showed no significant interaction of acrylamide dose level and Anakinra for microglial area and processes length within the dm-PFC and vm-PFC, suggesting no effect of Anakinra on the intensity of ACR-induced change in the area or process length of Iba-1-positive microglia.

DISCUSSION

The results of the study indicate that subcutaneous injection of Anakinra at a dosage of 25 mg/kg did not have any effect on the intensity of ACR-induced change in landing foot spread. The purpose of the study was to investigate the impact of inhibiting cytokines IL-1 on the neurotoxicity induced by acrylamide in mice. The idea for the current study was based on previously published data from our lab. The data showed that in male 10-week-old Wistar rats, a 5-week exposure to acrylamide led to inflammatory responses in the cerebral cortex. This was evident from the increased expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-18 at both the mRNA and protein levels (Zong *et al*., 2019b). In vitro studies using BV-2 microglial cells also confirmed a microglial inflammatory response upon exposure to acrylamide, as indicated by a time- (0-36 hr; 50 μM) and dose- (0-500 μM; 24 hr) dependent increase in mRNA expression of IL-1β and IL-18 (Zong *et al*., 2019b)

Recent studies have provided evidence of the involvement of acrylamide exposure in both central and peripheral neurotoxicity. These studies have shown that acrylamide exposure leads to an increase in the landing foot spread test, indicating motor dysfunction. Additionally, acrylamide exposure has been associated with an increase in the degeneration of noradrenergic axons in the cortex and prefrontal cortex (PFC) regions of the mouse brain (Davuljigari *et al*., 2021; Ekuban *et al*., 2021). According to a recent study by Ersoy *et al*. (2022), intraperitoneal administration with Anakinra at 50 mg/kg body weight has been shown to reduce neuropathic pain and prevent the neurotoxic effects of acrylamide on peripheral nerves. This is believed to be due to the analgesic, antioxidant, and anti-inflammatory properties of Anakinra. However, despite these findings and based on the studies mentioned earlier, our hypothesis that Anakinra would ameliorate ACR-induced neurotoxicity was not supported by the results of our study Moreover, our recent study using IL-1β knock out mice demonstrated that deletion of IL-1β exacerbate ACR-induced neurotoxicity (Fergany *et al*., 2023). Taken together with the present study, we conclude that inhibition of IL-1beta signal does not ameliorate ACR-induced neuropathy.

In contrast to the expected outcome, the treatment with Anakinra in our study potentiated the acrylamide-induced loss of body weight. This is interesting, as acrylamide itself has been shown to influence body weight. Previous research from our lab has demonstrated that acrylamide dose-dependently and significantly decreases body weight in mice, particularly at a concentration of 300 ppm (Davuljigari *et al.,* 2021). Regarding the effects of acrylamide on body weight. It appears that there is consistent evidence from previous studies showing that acrylamide exposure can lead to a decrease in body weight gain in maternal toxicity in rats and mice (Field *et al.,* 1990). Furthermore, oral administration of acrylamide in mice has been shown to decrease body

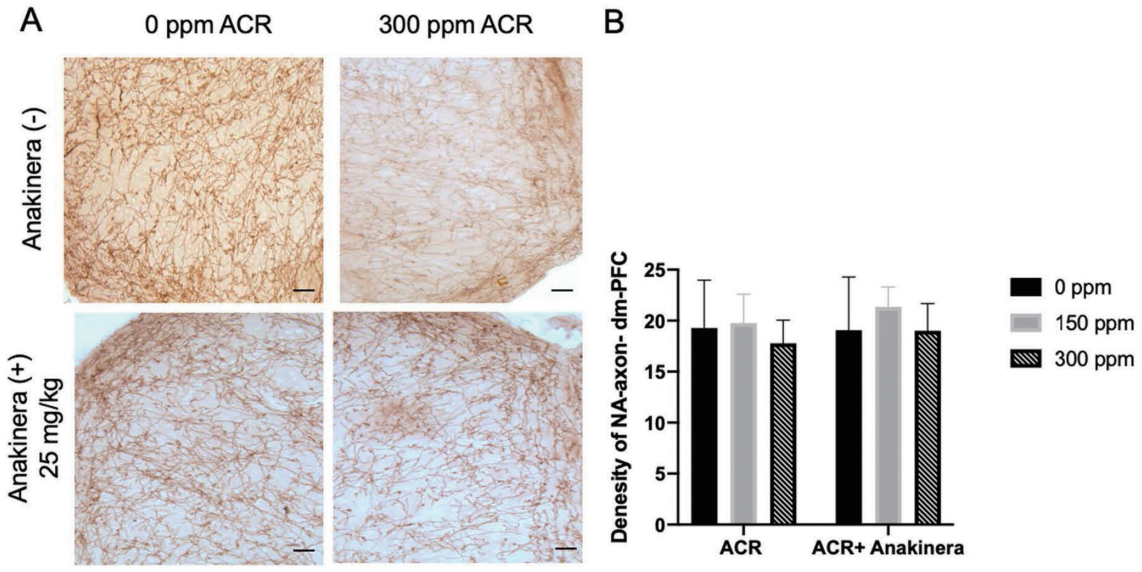

Fig. 3. Representative photomicrographs (A) and (B) density of noradrenaline transporter (NAT)-immunoreactive axons in the dorsal median prefrontal cortex (dm-PFC) following exposure to acrylamide, (0, 150, 300 ppm) or (0,150, 300 ppm ACR+ 25 mg/kg Anakinra). Data are mean ± SD. * $p < 0.05$, compared to the corresponding control. Scale bars = 40 µm; n = 4.

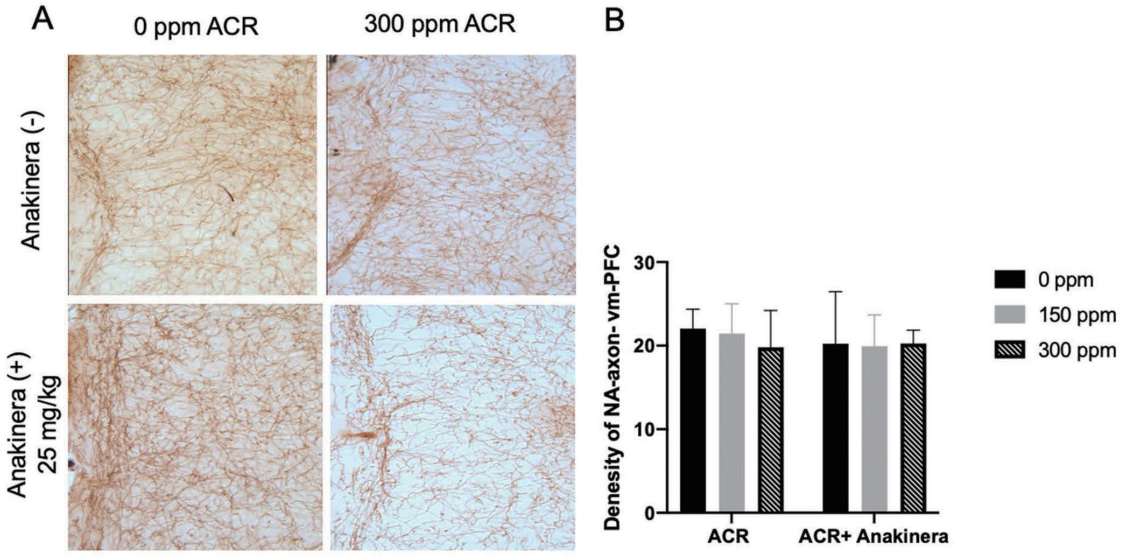

Fig. 4. Representative photomicrographs (A) and (B) density of noradrenaline transporter (NAT)-immunoreactive axons in the ventral median prefrontal cortex (vm-PFC) following exposure to acrylamide, (0, 150, 300 ppm) or (0, 150, 300 ppm ACR+ 25 mg/kg Anakinra). Data are mean ± SD. * $p < 0.05$, compared to the corresponding control. Scale bars = 40 µm; n = 4.

weight, with studies reporting this effect at various doses (Amirshahrokhi, 2021; Donmez *et al.,* 2020). Additionally, the association between chemical-induced neurotoxicity and body weight loss has been reported in studies on electrophile or gamma-diketone-induced neuropathy (Zhang *et al.,* 2020; Ichihara *et al.,* 2000; Takeuchi *et al.,* 1981). Collectively, the present study has certain implications that Anakinra should be used carefully for the

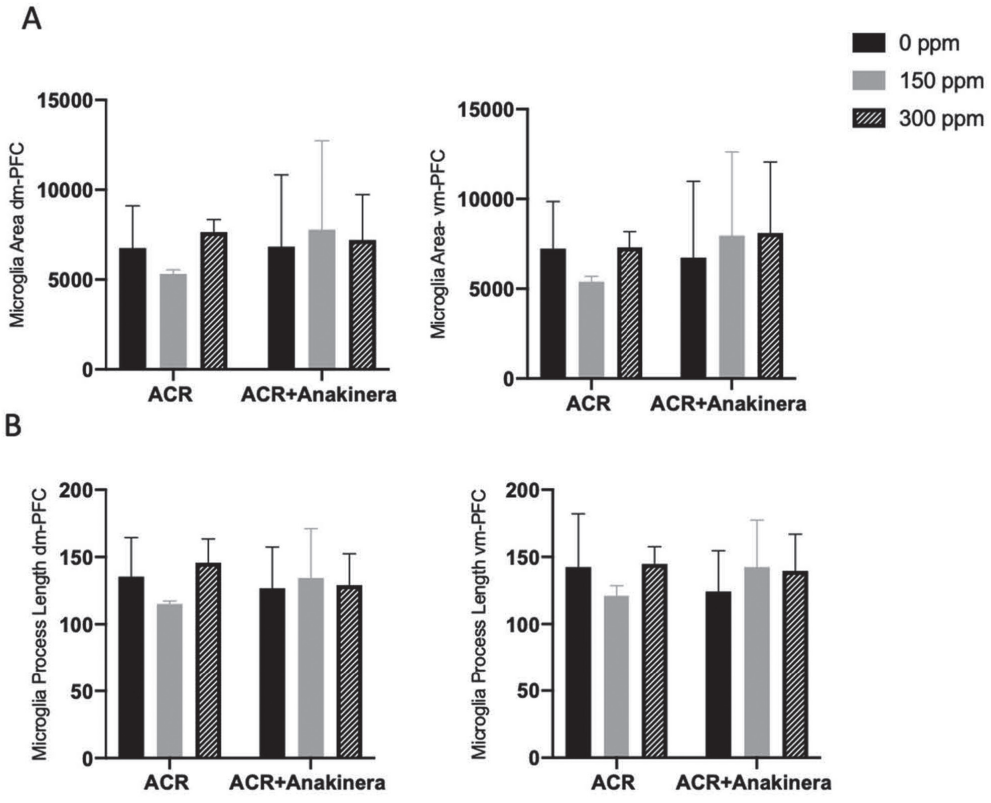

Fig. 5. Microglia area (A) and process length (B) in dorsal and ventral prefrontal cortex following exposure to acrylamide, (0, 150, 300 ppm) or (0, 150, 300 ppm ACR+ 25 mg/kg Anakinra). Data are mean ± SD. * $p < 0.05$, compared to the corresponding control.

patients of chemical-induced encephalopathy or presymptomatic disease stage.

The present study didn't show adverse effect of ACR on the density of noradrenergic axons in the prefrontal cortex, while it demonstrated effect of ACR on landing foot spread. This result suggests greater susceptibility of peripheral nerve than the central nervous system to ACR.

There is evidence suggesting the IL-1RA, can be transported actively across the blood-brain barrier (BBB). This transport has been observed *in vitro* and *in vivo* studies (Gutierrez *et al.,* 1994). Anakinra has been found in high concentrations in plasma and CSF and has been shown to penetrate brain tissue in areas where the blood-brain barrier is compromised, such as in cases of blood-brain barrier breakdown in MACO (Greenhalgh *et al.,* 2010). Additionally, IL-1RA has been reported to passively transport across the blood-CSF barrier in cases of subarachnoid hemorrhage (SAH) (Galea *et al.,* 2018). This data suggests that Anakinra may have the ability to reach the brain and potentially have a protective effect in various central nervous system disorders.

The dose of 25 mg/kb body weigh in the present study is rationalized by previous studies in human cases and experimental systems. According to a study by Jang *et al*. (2022) in refractory cerebral autoinflammatory-autoimmune diseases, a dose of 100 mg of Anakinra per person was found to be a therapeutic option in these conditions. In a case study involving a patient with Neonatal-onset multisystem inflammatory disease (NOMID), it was observed that the median of IL-1Ra levels in the cerebrospinal fluid (CSF) increased from 211 pg/mL before treatment to 1,136 pg/mL after 3 months of Anakinra treatment at 1 to 2 mg/kg body weight (Goldbach-Mansky *et al.,* 2006). Additional outcomes observed in the NOMID patient treated with Anakinra. It appears that the administration of Anakinra resulted in a significant decrease in the severity of various NOMID manifestations. This includes a reduc-

tion in elevated intracranial pressure, leptomeningitis, and neurosensorial hearing loss. Additionally, the treatment was associated with a decrease in cerebrospinal fluid (CSF) levels of IL-6. According to a study by Kenney-Jung *et al*. (Kenney-Jung *et al.,* 2016), Anakinra was administered at 5 mg/kg body weight twice daily subcutaneously to a young patient with a severe seizure disorder associated with febrile infection-related epilepsy syndrome. The patient experienced recurrent seizures daily, but with the daily subcutaneous administration of Anakinra, the frequency of seizures progressively decreased and eventually ceased. In experimental systems, the study by Martin *et al*. (1994), systemic administration of 100 mg/kg IL-1 receptor antagonist before and/or after hypoxic exposure was found to be protective against brain insult. Similarly, Taraschenko *et al*. (2021) demonstrated that subcutaneous injection of Anakinra at 25mg/kg twice daily for five days attenuated seizures and memory impairment induced by anti-NMDA receptor antibodies in mice.

It appears that while Anakinra has shown beneficial effects in various CNS disorders, our report on CNS toxicity indicates that daily administration of Anakinra at a dose of 25 mg/kg body weight did not ameliorate the neurotoxicity induced by ACR. Additionally, it seems that the Anakinra treatment in this case enhanced the ACR-induced body weight loss. These findings suggest that Anakinra may not be effective in mitigating the neurotoxic effects of ACR and could potentially exacerbate certain adverse effects.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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