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Research Paper

Zebrafish behavioral response to ivermectin: insights into potential neurological risk

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ABSTRACT

Ivermectin is a well-known and widely used anti-parasitic drug. Recently, *in vitro* data suggest anti-viral efficacy of the drug, albeit at much higher concentrations than currently approved. Despite warnings by several governing bodies, the (uncontrolled) human use of ivermectin has significantly increased during the COVID-19 epidemic. This study thus aimed to elucidate potential neurological risk of particularly the veterinary formulation of ivermectin in comparison to pure ivermectin. Zebrafish eggs (1hpf) and larvae (4dpf) were exposed to a range of concentrations of either pure ivermectin (IVM) or a veterinary formulation (V-IVM) for a period of 24 hours. Behavioral responses to both treatments were assessed at various timepoints using the pentylenetetrazol assay, the light–dark assay and a 5-day teratogenesis protocol. In addition, dissolution rates were calculated for both treatments. Acute responses of larvae at 4–<5dpf was similar for both treatments – a transient hyperlocomotion was followed by a general hypolocomotion (ANOVA dose effect, $P < 0.01$). Both IVM and V-IVM-treated larvae showed significant dose-dependent (ANOVA dose effect, $P < 0.0001$) decreases in responsiveness to repeated light–dark transitions, which again was more pronounced in IVM. These effects were maintained after 24 hours of exposure. In contrast, when ivermectin was administered prior to establishment of the blood brain-barrier in the teratogenesis protocol, V-IVM treatment was linked to more severe activity decline on <5dpf. Differences in dissolution rates cannot account for these differences. In conclusion, current data suggest significantly higher neurological risk of a veterinary formulation of ivermectin under conditions of penetration across the blood brain-barrier.

1. Introduction

The current COVID-19 pandemic has spurred a massive research effort into therapeutics and vaccines against the SARS-CoV2 virus in order to save lives [1]. Given the urgency of the need for therapeutics, drug repurposing formed a substantial portion of these efforts, although not all potential treatments were sufficiently assessed scientifically for this application. Ivermectin is one example of such a drug that gained significant attention during the pandemic.

Ivermectin was originally developed as a new class of drug to treat parasitic infections in the late 1970s [2]. Although initially used in veterinary medicine, ivermectin is currently approved for human treatment of parasitic infections such as onchocerciasis, lymphatic filariasis, strongyloidiasis and scabies in several countries [2], and recently as a topical treatment of moderate to severe inflammatory lesions of papulopustular rosacea in adult patients in South Africa [3].

Unfortunately, limited safety data is available on ivermectin. A 2020 meta-analysis which included six studies reported it to be safe and effective for anti-parasitic use in humans [2]. Doses of up to 800 μg/kg were tested in one of the studies, although the standard dose for treatment of parasitic infections is commonly accepted to be below 400 μg/kg [4]. However, even when administered according to the registered parasitic dose indications (prior to COVID-19), serious neurological adverse events were reported following large scale community-based ivermectin treatment campaigns against *Onchocerciasis volvulus* in Africa [5]. Numerous cases of serious neurological adverse events occurring with the use of ivermectin outside of the onchocerciasis indication were identified in VigiBase (an international

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pharmacovigilance database of suspected adverse drug reactions) in this study.

The expanded use of ivermectin as a therapeutic option for viral infections was recently suggested, following reports of *in vitro* activity against a broad range of viruses, including HIV, Dengue, Influenza and the Zika virus [6]. Ivermectin's inhibition of specifically importin α/β nuclear transport proteins, with no effect on a range of other nuclear import pathways, including those mediated by importin β1 alone, was already demonstrated in *in vitro* studies a decade ago [7]. Importantly, antiviral activity of ivermectin towards both HIV-1 and dengue virus, both of which are strongly reliant on importin α/β nuclear import, with respect to the HIV-1 integrase and non-structural protein 5 (NS5) polymerase proteins, respectively, were reported from these *in vitro* studies. However, ten years later ivermectin is still not prescribed as an anti-HIV treatment. Recently Caly *et al.* proposed the possibility of using ivermectin to combat COVID-19[7]. The authors demonstrated that ivermectin possesses the ability to inhibit the replication of SARS-CoV-2 *in vitro*, by resulting in a 99.8 % reduction in viral RNA after 48 hours [8].

However, for ivermectin to reach the effective antiviral concentration reported in the aforementioned studies (about 2–10 μM), administration of extremely high oral doses (several grams per day) will be required, which poses a significant risk of toxicity to the patient [9]. Despite the concerns regarding possible toxicity, and largely due to uninformed communication on social media, ivermectin took the world by storm as a possible candidate for COVID-19 prevention and treatment [10]. Several studies have claimed benefit of use, but were criticized by the scientific community as poor-quality and misleading research [11,12].

In stark contrast to the optimistic pre-clinical and social media claims of the anti-viral effect of ivermectin, a recent randomized control trial showed no benefit for ivermectin (≈300-400 ug/kg over 48 hours) in treating COVID-19 [13]. In fact, patients who received ivermectin required invasive mechanical ventilatory support earlier in their treatment compared to patients who were not using ivermectin [13]. Also in 2021, an article published in the Cochrane Library Systematic Reviews concluded that there was a lack of high quality research data to support the safety and efficacy of ivermectin, and the authors recommended that ivermectin should not be used outside of "well-designed randomized trials" [14]. During this time, ivermectin had not been approved for the prevention and treatment of SARS-CoV-2/ COVID-19, rather, the FDA had already issued a warning against its use for the said treatment in April 2020 [15]. Despite the recommendations against its use for treating COVID-19, utilization of ivermectin continues to be used by the public and health care services.

An alarming result of the reluctance of health care authorities to approve and prescribe ivermectin as potential COVID-19 treatment, is the widespread reports of the use of ivermectin-based veterinary products for prophylaxis or treatment of COVID-19 in humans, e.g. as highlighted in a statement released by the South African Health Products Regulatory Authority (SAPHRA) in 2021 [16]. In accordance with the 2020 FDA warning, SAPHRA echoed statements from CERTARA, and Pharmacometrics Africa, that *'Preliminary findings suggest that standard doses of ivermectin would not result in efficacious concentrations, and that extraordinary doses to achieve efficacious concentrations may result in unacceptable toxicity in COVID-19 patients."* [16].

Given the widespread use of ivermectin, despite numerous reports of deleterious effects and a relative lack of safety data, it is imperative to elucidate potential risks associated with ivermectin toxicity, to both inform the lay public on potential risks and contribute to well-informed patient management strategies. The use of different formulations of ivermectin available on the market, which may exhibit a variety of pharmacokinetic characteristics, and thus potentially different absorption-extrusion activity or different dissolution properties of the drug, may further impact on its risk profile. Given this high risk to especially the uninformed, desperate lay person, more research is clearly warranted.

In terms of risk profiling priority, the side-effects reported in the periphery – such as nausea, vomiting, rash, diarrhoea, hypotension and even hepatitis – is readily identified and thus will likely not go unnoticed or untreated. However, neurological adverse effects – suggested by reports of headache, dizziness, seizures, confusion, loss of consciousness/depressed level of consciousness, abasia, tremor and coma may indicate neurological damage that cannot be reversed or easily managed [16,17]. In the context of COVID-19 specifically, the SARS-CoV-2 virus was shown to cause – either directly or as result of the associated cytokine storm – significant disruption of the blood brain barrier [18], which would potentially increase access of administered drugs, such as ivermectin, to the brain, thus exacerbating potential neurological adverse effects of ivermectin. The aim of this study was therefore to investigate the potential neurological risk associated with ivermectin treatment by comparing a pure compound intended for human-use and a veterinary formulation against a control group, in zebrafish larval models.

2. Materials

Ivermax veterinary preparation (available as 1 % ivermectin m/v in glycerol formal and propylene glycol) (V-IVM) was purchased from Cipla Agrimed (Pretoria, South Africa). Pure ivermectin (IVM) [Batch#: I202106044] was a gifted by Idexis Compounding Specialists (Cape Town, South Africa). Using Raman spectroscopy (Supplementary Material) and HPLC, presence of ivermectin was confirmed in both samples. Assuming 100 % purity of ivermectin in IVM, the relative purity of V-IVR was 82.12 ± 2.27 %.

Sodium chloride, potassium chloride, calcium chloride, magnesium sulphate and pentylenetetrazol salts were purchased from Sigma-Aldrich (Now Merck, Germany). Chromatography grade acetonitrile, chromatography grade methanol and hydrochloric acid (32 %) were purchased from Sigma-Aldrich (Johannesburg, South Africa) and ultrapure water with a resistivity of 18.2 MΩ was obtained from a Lasec Purite water purification system (Johannesburg, South Africa).

3. Methods

3.1. Zebrafish experiments

Ethical considerations: All experiments were performed in wild-type zebrafish larvae within 5 days post-fertilisation (<5 dpf). All protocols were ethically cleared by the Stellenbosch University Animal Research Ethics Committee (ACU-2019-11820). Eggs were obtained within 1 hour of spawning from the Zebrafish Research Unit in the division Clinical Pharmacology in the Department of Medicine (Faculty of Medicine and Health Sciences, Stellenbosch University). Eggs and larvae were maintained in E3 embryo medium at 28 °C, with a 14/10 light/dark cycle and refreshed daily according to standard protocols.

Pentylenetetrazol (PTZ) assay: IVM has been reported to act on the $GABA_A$ receptor [19]. The PTZ assay was employed to assess the potential of ivermectin to modulate GABA receptor signalling [20]. PTZ is a known seizure-inducing agent and has demonstrated to exert its effects by acting as a GABA receptor antagonist [20]. Larvae were pipetted into 96-well plates in E3 medium. The assay consisted of the following treatment groups; control (E3 medium), PTZ-treated (10 mM), V-IVM and IVM-treated (doses between 0.1 and 10 μg/mL) as well as a combination treatment (10 mM PTZ and 4 μM diazepam) group. Diazepam, a known GABA agonist, was used as assay validation control. Behavioral tracking was performed for 20 minutes, starting immediately after the addition of treatments, using the Noldus DanioVision tracking system, with an acquisition rate of 25 frames per sec.

Activity data was analysed using Noldus Ethovision software, applying a smoothing threshold of 0.2 mm.

Light-dark transition assay: The effects of ivermectin on behavioral responsiveness to bright light–dark transitions, a protocol known to result in anxiety-like behaviour [21], was also assessed. Zebrafish larvae were exposed to IVM and V-IVM (0.1–10 μg/mL) by immersion, and activity recorded acutely, as well as after 3hr and 24hr of treatment exposure. In each trial, larval activity was recorded for a total period of 30 minutes consisting of 10 minutes of basal activity, followed by 2 repeated light–dark cycles (each 5 minutes bright light followed by 5 minutes of darkness).

Teratogenesis assay: To evaluate potential teratogenic effects of IVM and V-IVM, zebrafish eggs were exposed to IVM and V-IVM (0.1–0.5 μg/mL) in E3 medium by immersion for 24 h, starting at 6 h post fertilisation (6 hpf). Following the 24 h treatment, E3 medium was refreshed to remove IVM and V-IVM, and again daily thereafter until <5 dpf (118 hpf). Basal behaviour (30 min) and responsiveness to a light–dark transition (5 min bright light, 5 min dark) was assessed using the DanioVision activity tracker and Ethovision analytical software.

Statistical analysis: Zebrafish larval assays were conducted using at least n = 12 per treatment group. Activity tracks over time are presented as means ± standard error of the mean (SEM) of data binned into 1 min bins, and total distance data used for statistical analysis, as means ± standard deviation (SD). GraphPad Prism v.8 was used to construct figures as well as for statistical analyses. Data were analysed using ANOVA followed by Tukey's multiple comparisons test.

3.2. Dissolution rate comparison

Dissolution testing: The dissolution rates of IVR and V-IVR were investigated in a pH 1.2 buffered aqueous medium using a USP II (paddle) dissolution setup. Prior to each dissolution experiment, each of the six dissolution vessels was filled with 900 ml of the dissolution medium. The dissolution medium was heated to 37.5 ± 2 °C and was maintained at that temperature throughout. The dissolution medium in each dissolution vessel was agitated using a paddle rotational speed of 100 rpm. To each vessel, an amount equivalent to 12 mg IVR was added, in accordance with the once-off daily dose of ivermectin currently indicated for human use. At predetermined time intervals (2, 5, 10, 20, 30, 40 and 60 min), 2 mL of the dissolution medium was withdrawn and subsequently filtered through a 0.2 μm nylon filter into high-performance liquid chromatography (HPLC) vials for analysis. After each withdrawal, the same volume of dissolution medium was replaced with heated dissolution medium, to ensure that reaction conditions were maintained throughout the drug dissolution process.

High-performance liquid chromatography (HPLC): The concentration of dissolved IVR was determined using a Knauer Azura (Berlin, Germany) HPLC system equipped with a 2.1 L diode array detector (set at 254 nm), 6.1 L autosampler, quaternary pump and column thermostat. The mobile phase consisted of 15:34:51 v/v ultrapure water: methanol:acetonitrile. A Phenomenex® Kinetex® C_{18} column, 250 × 4.6 mm, (Torrance, USA) was employed. The injection volume was 10 μL and the flow rate was 1.5 mL/min. A correlation coefficient (r^2) of 0.999 was obtained for this analytical method.

4. Results

In the first experiment, the effect of ivermectin on GABA receptor signalling was assessed. Activity of ivermectin-treated larvae were compared to that of PTZ-treated larvae – where GABA-receptor antagonism is known to result in hyper-excitation-associated hyperlocomotion.

When considering the activity tracks (Fig. 1a, c) and quantified activity data (Fig. 1b, d), the PTZ assay is validated by the expected behaviour larvae treated with PTZ and those treated with a combination of PTZ and diazepam. The PTZ-treated larvae exhibited immediate and prolonged hyperlocomotion relative to control larvae, which resulted from the relative absence of GABA signalling, while those treated with the combination treatment, exhibited low activity and relative unresponsiveness to PTZ, which is known to result from the sedative effect of diazepam, a GABA agonist.

In a manner similar to that of PTZ, the highest dose of ivermectin (both V-IVM and IVM) also resulted in a seizure-like hyperlocomotion response, followed by hypolocomotion. Of interest, the activity trajectory and onset of lethargy seemed to occur significantly faster in larvae treated with IVM than those treated with V-IVM formulation (Fig. 1a, c). At lower doses, V-IVM-treated larvae exhibited activity levels similar to that of controls (Fig. 1b). However, there was a tendency for total activity to decrease in a dose-dependent manner across all doses of IVM (ANOVA main effect of dose, $P < 0.01$), reaching statistical significance at the highest dose assessed (Fig. 1d), which suggests a potential excitotoxicity/seizure effect of the highest dose of IVM. At this dose, the IVM-treated larvae exhibited significantly lower activity levels when compared to the same dose in V-IVM (Fig. 1d).

The behavioral response to the anxiety-inducing light–dark transition protocol was also evaluated as an indicator of cognitive function after exposure to ivermectin (pure IVM and V-IVM). Assessments were carried out at three time points after initiation of treatment by immersion: immediately, after 3 h and after 24 h. At the acute time point (Fig. 2a, d), control animals had not yet settled to a stable baseline activity level at the start of the lights-on period, which masked the startle response seen at the time point when the bright white light is switched on for the first time – the response is only partly visible in the IVM group at time point 10 min (Fig. 2d). Of interest, this allowed the observation of significantly decreased activity in all IVM and V-IVM treated larvae, independent of dose. For both treatments, the startle response was observed in the lower (0.1 and 0.5 ug/ml) doses at the acute time point, but not in the higher (1 and 10ug/ml) doses. Of interest, only the lowest dose retained this startle response at 3hr (Fig. 2b,e), with a similar outcome observed for both IVM and V-IVM. In contrast, at 24hr (Fig. 2c,f), the startle response appeared to have been recovered in the V-IVM-treated larvae for the 0.5 ug/ml dose, but not in the IVM group.

In terms of the hyperlocomotion response, only the two lowest doses of V-IVM did not result in inhibition of the immediate hyperlocomotion response, with both higher doses resulting in an immediate relative unresponsiveness (visible as similarly low activity levels in both light and dark cycles) (Figs. 2a and 3a), which expanded to the 0.5ug/ml dose by 3hr (Figs. 2b and 3b). As with the startle response, larvae treated with 0.5 ug/ml V-IVM again regained responsiveness after 24 hr, when both 0.1 and 0.5 ug/ml dose-treated larvae exhibited normal anxiety behaviour (Figs. 2c, 3c). A similar effect was observed in the IVM-treated groups, but again, this formulation seemed to exhibit higher potency: immediately after treatment, only the lowest dose did not have inhibitory effects (Figs. 2d, 3d), and even the lowest dose was associated with limited responsiveness at 3hr (Figs. 2e, 3e), from which larvae were unable to recover by 24hr (Figs. 2f, 3f).

In contrast to the behavioral assessments on 4–5 dpf after acute ivermectin treatment, in terms of potential teratogenic effects, although no morphological abnormalities were observed, all V-IVM treatment doses – with the exception of the lowest dose employed – significantly reduced basal activity status (ANOVA main effect of dose, $p < 00001$) and was associated with a relative absence of anxiety-associated hyperlocomotion response in the light–dark assay on day 5 post fertilisation, when compared to untreated controls (Fig. 4a,b). Interestingly, despite their relative lethargy, larvae in all dose groups still exhibited a similar, normal startle response to sudden bright light (min 30, Fig. 4a). In contrast, the IVM treatment doses appeared to

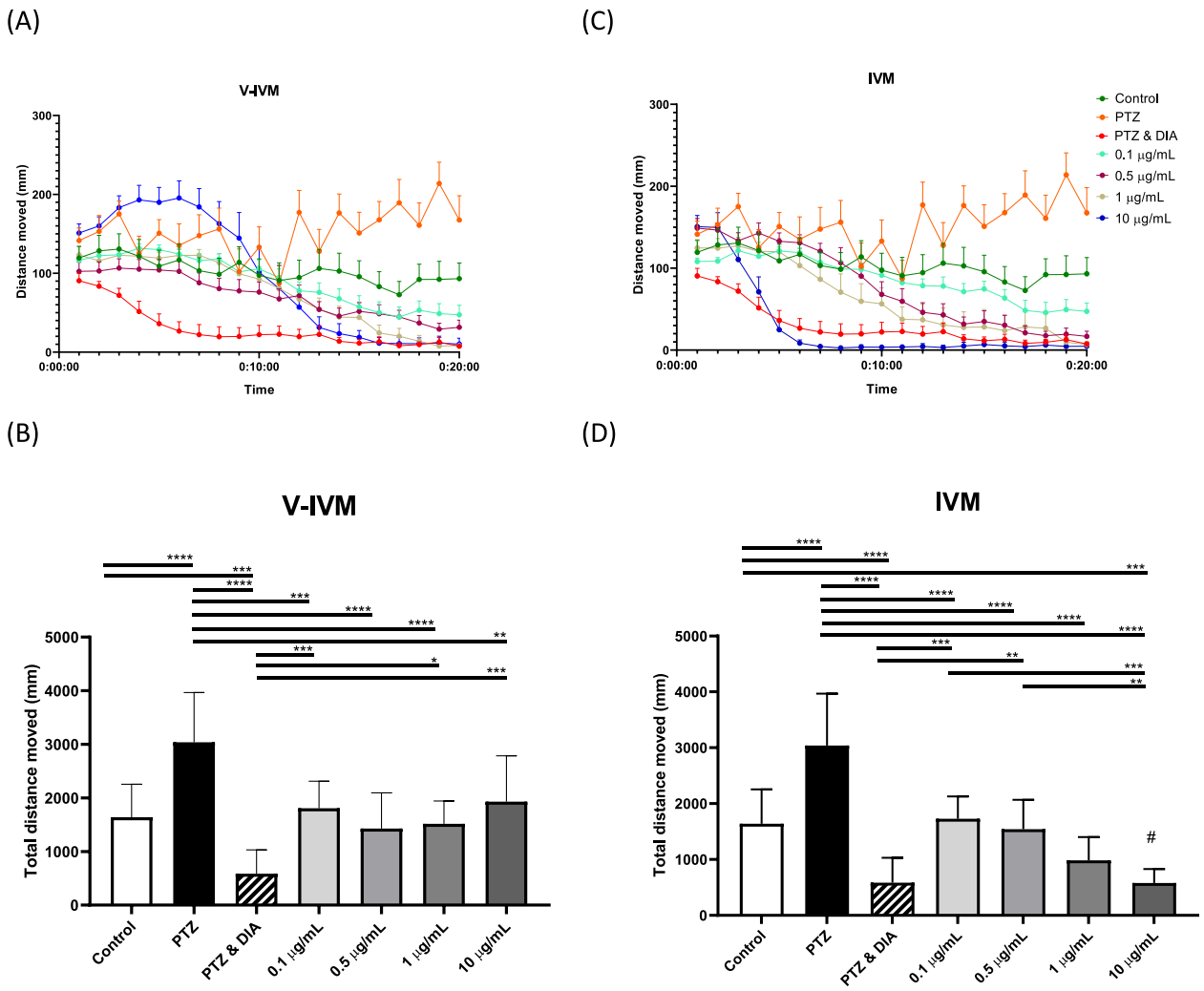

Fig. 1. Behavioral response of zebrafish larvae (<5 dpf) treated with veterinary (V-IVM) and pure (IVM) formulations of ivermectin. Pentylenetetrazol (PTZ, 10 mM) was used as standard excitotoxicity agent, and diazepam (DIA, 4uM) as standard rescue treatment. Activity data is presented as 1-minute bins (means and SEM) in (a) and (c), while total activity (means and SD) is presented in (b) and (d) for V-IVM and IVM respectively. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; #, Significant difference between IVM and V-IVM at this dose, $p < 0.001$).

have much less of an effect (ANOVA main effect of treatment, $p < 0.05$), with only the 0.4 µg/mL dose reaching statistical significance suggestive of a negative effect on embryonic and larval development in this context (Fig. 4b,d).

The rate at which the ivermectin reached the solubilised state, as well as the percentage ivermectin that was able to dissolve, was assessed using dissolution testing (Fig. 5). IVM exhibited a significantly faster dissolution rate than V-IVM, with approximately 50 % more IVM reaching the solubilised state in 5 minutes. For both IVM and V-IVM, a dissolution plateau was reached after approximately 20 minutes, with IVM exhibiting a 3.17 % higher dissolved ivermectin concentration than V-IVM. The initial 'burst' in the dissolved ivermectin concentration is a common phenomenon observed with dissolution testing, especially in instances where the drug exists in a metastable solid-state form [22].

5. Discussion

Current data, generated for doses of ivermectin in line with those reported to be effective as anti-viral treatment in *in vitro* models, suggest significant cause for concern in terms of neurological risk, should it be possible to achieve these high doses in humans.

Firstly, current data demonstrating an acute (transient) hyperlocomotion response to ivermectin (both IVM and V-IVM) that suggests a GABA antagonistic effect of ivermectin, are in line with existing literature from pharmacological models reporting irreversible binding of ivermectin to the $GABA_A$ receptor [19] – the major inhibitory neurotransmitter receptor in the brain – as well as potentially to the $GABA_B$ receptors, as demonstrated in rodents *in vivo* [23]. In terms of mechanisms other than GABA signalling inhibition, ivermectin-associated hyperlocomotion in rodents have also been linked to increased striatal activity of serotonin and dopamine [24]. Of further relevance to our topic, dysregulated (increased) serotonin and dopamine activity in the striatum – the hub for control of movement – has been linked to neuropsychiatric disorders including Parkinson's disease, Gilles de la Tourette syndrome and impulse control disorders [25].

Secondly, current data consistently demonstrate the brief hyperlocomotion to be followed by hypolocomotion, especially evident in response to the added stressor of bright light exposure in zebrafish larvae. These data are again in line with a study reporting hypolocomotion in rodents exposed to both ivermectin and stressful stimuli [25]. A potential mechanism at play in this context has been illustrated in a *C.elegans* model, where the frequency of channel opening of a levamisole-sensitive nicotinic receptor (L-AChR) was irreversibly

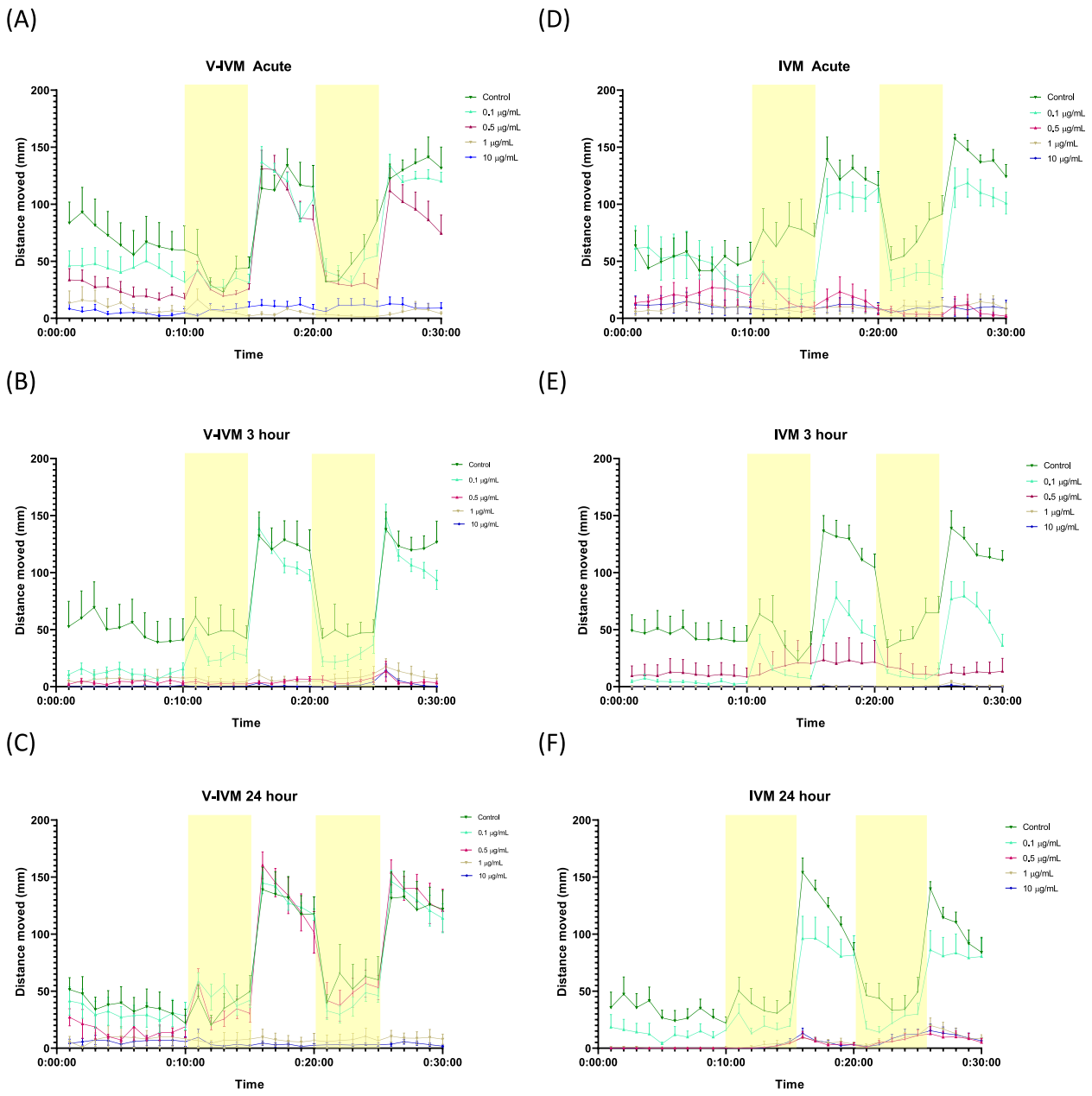

Fig. 2. Altered responsiveness to light–dark transitions in zebrafish larvae (<5dpf) treated with different concentrations of a veterinary formulation IVM (left panel) or pure IVM (right panel). Yellow blocks indicate periods of bright light exposure. Behavioral assessments were repeated immediately after treatment (a and b), as well as after 3hr (c and d) and 24hr (e and f) of immersion exposure. Data were acquired as 25 frames/second and binned into 1-minute bins and are presented as mean ± SEM.

inhibited by ivermectin [26]. Dysregulation of AChRs signalling has been implicated in mood disturbances such as anxiety and depression in humans [27]. A role for ivermectin at the level of these receptors may thus explain the hypolocomotion, but potentially also at least in part the hyperlocomotion, observed in the current study. Thus, in terms of the effects of ivermectin at neurotransmitter receptor level, clearly more than one receptor is implicated, while different time courses or dose-specificity in terms of the effect achieved, as well as the confounding effects of stressors, further contribute to the net effect observed. Nevertheless, current data clearly indicates the need for caution in the context of ivermectin use as antiviral treatment in humans, especially considering the high doses required for antiviral activity.

An interesting observation was the loss of the startle response at the time of first onset of bright light in the light–dark transition assay, in larvae exposed to higher doses of ivermectin. In non-mammal vertebrates, one particular neural circuit – the Mauthner circuit – is crucial for rapid decision making when faced with a threat [28]. This circuit is known to be responsible for the startle response in zebrafish [29]. Although humans do not have Mauthner cells, it is of relevance to the current topic to note that Mauthner cell desensitisation to threatening stimuli – as seen here by the loss of the startle response – has been linked to increased clustering and activity of glycine receptors in zebrafish Mauthner cells [30]. Furthermore, exacerbated startle (hyperekplexia) in humans is known to result from decreased signalling through glycine receptors, suggesting similar signalling role players in zebrafish and humans [31]. Given the fact that ivermectin has been reported to be a high potency glycine receptor agonist [32], it is thus possible that ivermectin may affect decision-making in

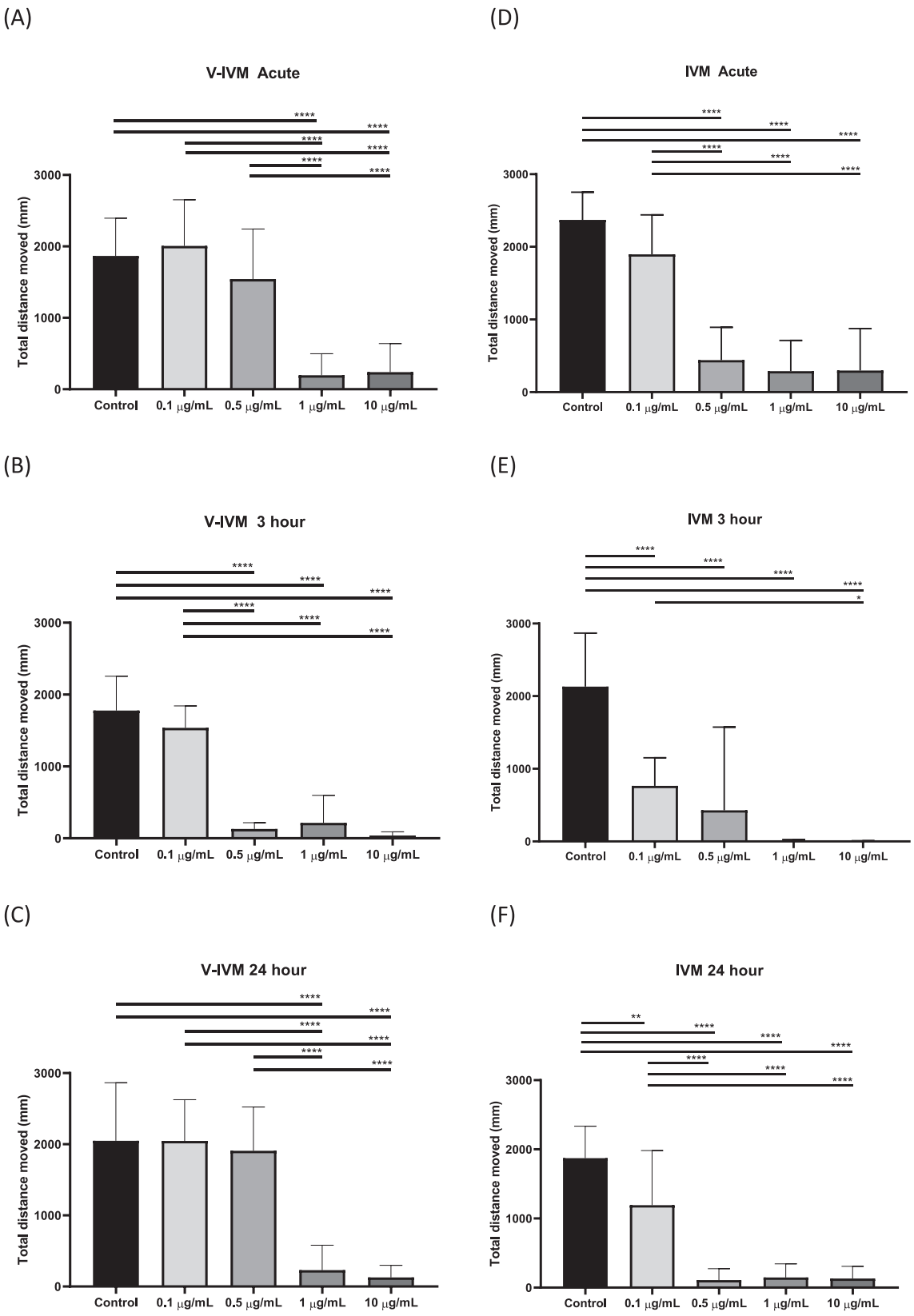

Fig. 3. Quantification of the hyperlocomotion response to light–dark transitions in zebrafish larvae after treatment with a veterinary formulation of ivermectin (V-IVM)(left column) or pure ivermectin (IVM) (right column) for up to 24 hours. Data are presented as mean ± SD. (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$). ANOVA indicated a main effect of treatment dose ($p < 0.0001$) for both treatments at all time points assessed.

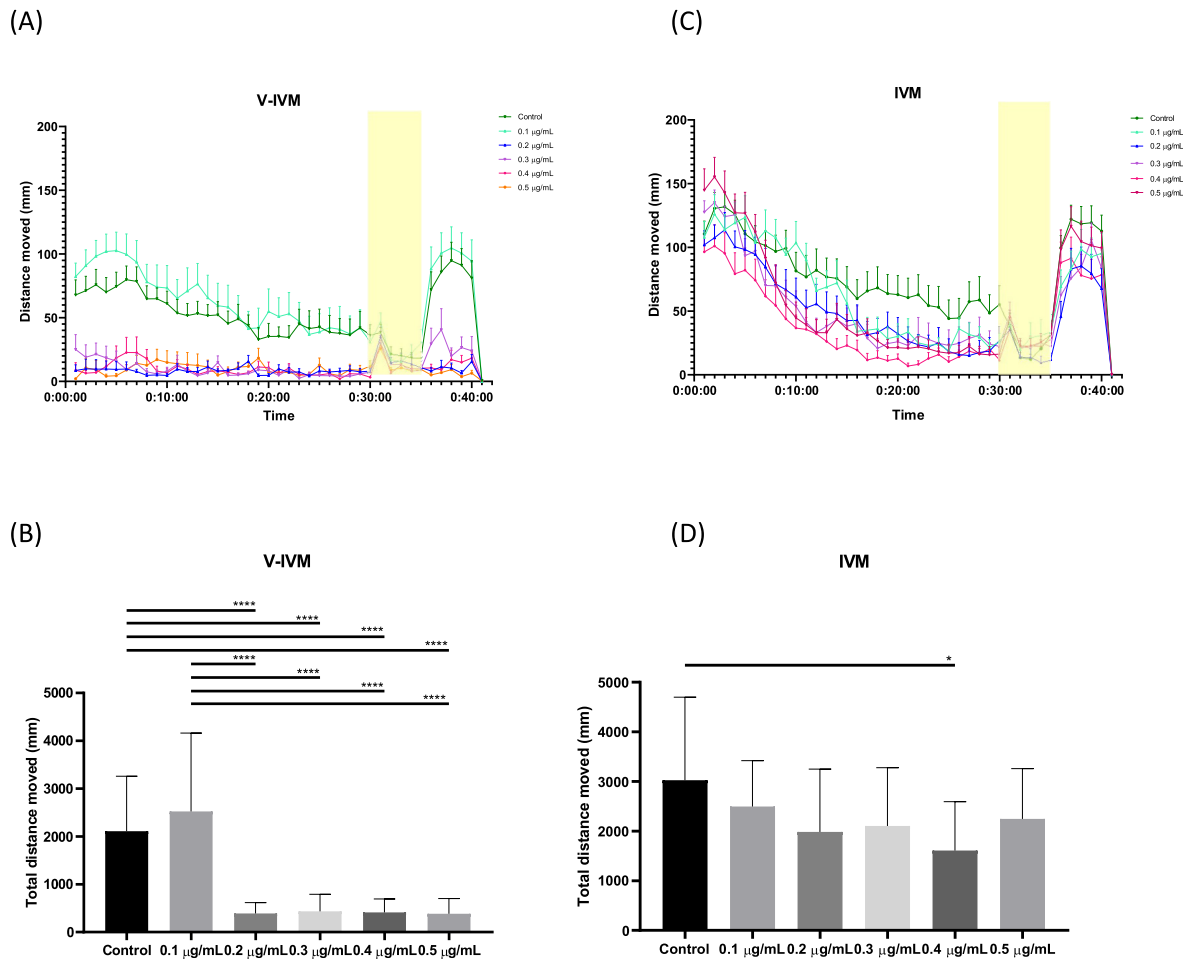

Fig. 4. Basal activity and responsiveness to anxiety-inducing light–dark stimuli, of <5dpf zebrafish larvae after 5-day treatment with VIVM and IVM. Activity over time is presented in 1-minute bins in (a) and (b), with total movement means and standard errors of the mean in (c) and (d). Data are presented as mean ± standard error of the mean (a & b) and mean ± standard deviation (c & d). (*, $p < 0.05$; ****, $p < 0.0001$).

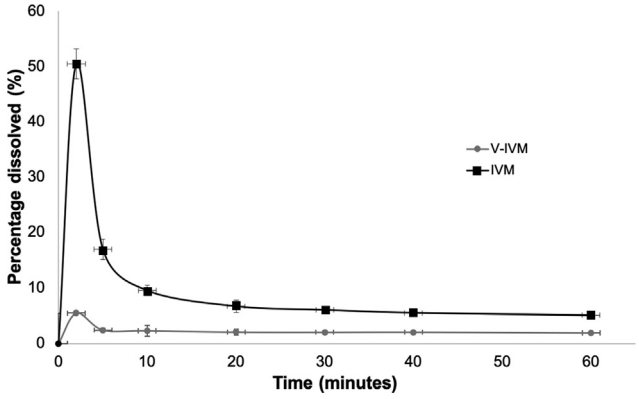

Fig. 5. An overlay of the percentage of ivermectin dissolved from V-IVM vs IVM.

humans similarly than in zebrafish, albeit via somewhat different neural circuits.

In terms of the differences in risk profile between IVM and V-IVM, current data did not consistently favour one formulation above the other. In the activity assays where ivermectin was administered at 4 dpf (which is after establishment of the blood brain-barrier) the veterinary formulation seemed to elicit slightly less severe effects than the human formulation. However, when a single administration of ivermectin was applied at 6 hpf (before establishment of the blood brain-barrier), the veterinary formulation showed significantly greater effects from which larvae could not recover, as illustrated by significantly lower activity levels and responsiveness to a bright light stimulus at <5 dpf.

In terms of potential reasons for these differences, as confirmed by further characterisation and dissolution properties of V-IVM and IVM, V-IVM seemed to contain a somewhat different solid-state form of ivermectin than IVM. More analytical techniques are necessary to confirm the exact solid-state form, but this alone does not explain the inverse risk profiles observed in acute vs longer-term treatment models in the current study. Rather, the somewhat lower purity of the V-IVM, together with its poorer solubility, may explain the seemingly lower risk profile in the acute (<24 hr) experiments. However, a potential longer period of sustained release of the V-IVM (due to its poorer solubility) cannot explain the poorer outcome in the teratogenesis assay, as exposure time to ivermectin treatments were still limited to 24 hours. Rather, the increased access across a not yet intact blood brain-barrier, may indicate a higher risk for undesired neurological side effects in V-IVM than IVM. In the context of SARS-CoV-2 infection, where known interactions between the virus and the blood brain-barrier were illustrated to compromise blood brain-barrier integrity [18], exposure of the brain to ivermectin will be increased in a manner similar to the scenario in the teratogenic study. This supports our interpretation of a poorer neurological risk profile for V-IVM, especially in the context of viral infection. In addition, direct effects caused by other

constituents of V-IVM (glycerol formal and propylene glycol) seem unlikely to be a confounder, given the high dilution factor (x100 000) used in preparation, which achieved levels far below reported tolerable levels of these substances [33,34]. However, a potential vehicle effect cannot be excluded. Studies on drug (or solvent) tolerance are typically conducted on zebrafish at 4dpf, i.e. after establishment of a blood brain-barrier. Thus, exposure in the teratogenesis assay may have increased brain exposure to these solvents – such a scenario has not been tested comprehensively for solvent tolerance. Nevertheless, this is not a limitation in the current study, where the aim was to specifically assess neurological risk of administration of solvent-containing formulations in the potential presence of (SARS-CoV-2) viral infection. Rather, this result warrants further caution against the use of impure formulations of ivermectin regardless of disease context.

Our interpretation of behavioral responses to ivermectin in zebrafish is also in line with literature focused on neurotoxicity risk dependent on the absorption-extrusion activity of the drug from the gastrointestinal tract and blood brain-barrier, which is regulated by P-glycoprotein (P-gp), environment-susceptible genes coding for P-gp, such as ABCB1 (also known as multi-drug resistant express the ABCB1 gene, they do express the homologs ABCB4 and ABCB5, and ABCB4 has indeed been demonstrated to phenocopy P-gp function in zebrafish [36].) Genetic mutations in this context may therefore further increase the risk of penetration of ivermectin across the blood brain barrier, e.g. mutations in the MDR1 gene have indeed been linked to increased penetration of ivermectin into the CNS [37]. The MDR1 transporter plays a crucial role in ivermectin efflux at low drug concentration, with a slow transport rate [35,38]. However, saturation of the MDR1 transporter (MRP1), as well as other transporters like MRP2 and MPR3, occurs at higher drug abundance - IC50 values of 0.1–2.5uM has been reported for ivermectin inhibition of P-gp-mediated transport [17,38]. This results in increased ivermectin transport across physiological barriers, including the blood brain-barrier [35]. Thus, in our opinion, given the detrimental effects illustrated here for V-IVM in the teratogenesis study in particular, V-IVM use in humans should not be supported.

6. Conclusion

Taken together, current data suggest that the veterinary formulation of ivermectin may have lower availability after acute administration when compared to pure ivermectin. However, V-IVM may pose a greater risk for undesired longer-term neurological outcome, especially in the context of viral disease, when virus-drug interaction may further compromise blood brain-barrier integrity. Given these risks, the origins of side-effects reported after anti-parasitic use of ivermectin should probably be revisited as well, as longer-term risk even after much lower levels of ivermectin, has not been determined.

CRediT authorship contribution statement

Yigael Powrie: Conceptualisation, Investigation, Methodology, Data curation, Formal analysis, Visualization, Writing – review and editing. **Morné Strydom:** Writing original draft. **Marique Aucamp:** Investigation, Methodology, Data curation, Formal analysis, Visualization. **Natalie Schellack:** Writing – review and editing. **Vanessa Steenkamp:** Writing – review and editing. **Carine Smith:** Conceptualisation, Investigation, Methodology, Data curation, Formal analysis, Visualization, Project administration, Supervision, Project administration, Supervision, Writing original draft.

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Ethics statement

Zebrafish larvae < 5dpf are not seen as sentient animals and protocols using them at this early stage are thus exempt from ethical approval. However, despite this global practice, we submitted and obtained ethical clearance for all protocols from the Stellenbosch University Animal Research Ethics Committee (ACU-2019-11820).

All protocols were executed in line with good laboratory and good laboratory animal practices, as prescribed by the South African National Standard (SANS) on animal research and the South African Medical Research Council.

Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.medidd.2022.100141.

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