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BEHAVIOURAL ANALYSIS OF POTENTIAL NEW APPROACH IN MODELLING SCHIZOPHRENIA USING ZEBRAFISH

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Abstract
Schizophrenia spectrum disorders are one of the most debilitating disorders as it severely impacts the day-to-day life of the individual. Therefore, during recent years, using zebrafish to model schizophrenia has become a reliable and popular choice due to the similarities in terms of genetics and brain structure to that of the human brain. Often times, the approach is to use ketamine in order to induce the positive symptoms of this disorder in zebrafish. However, this method may be lacking since it only mimics the positive symptoms, represented by delusions, hallucinations, which are not always the standard in terms of symptoms. Therefore, in the present study we have decided to approach this animal model from a different perspective by administrating methionine to replicate the negative symptoms described through increased anxiety, decreased social life and skills, and also the combination of the two substances with the purpose to get as close as possible to the way the disorder is manifested in humans. Our results support the idea that both ketamine and methionine are reliable options to induce their respective symptoms whereas the combination of the two substances does indeed lead to different behaviors compared to the individual groups.

Keywords: animal model, methionine, schizophrenia, zebrafish.

1. INTRODUCTION

Schizophrenia is, without doubt, the most debilitating and severe psychiatric disorder (Stefan et al., 2002). Contrary to the popular belief, schizophrenia is not a new disorder, nowadays is just better understood and actually diagnosed. Schizophrenia is characterized by delusions, hallucinations, disorganized speech and thought processes, severe disorganization and abnormal motor behavior (including catatonia), and negative symptoms for an extended period of time within a month (American Psychiatric Association, 2013).

Although schizophrenia is a disorder that has been studies for decades, a consensus has still not been reached regarding the complex etiology of this disorder, but it is general accepted that it is determined by a mix of factors, both internal (genetic) and external (the environment) (Patel et al., 2014). These factors can influence the prevalence of the disorder in the general population, the time of debut and the severity of the symptoms (Petrova & Khvostikova, 2021). More reason why there

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is an absolute need of animal models that are able to mimic as close as possible the manifestations observed in humans in order to develop new therapies and better diagnosis tools.

Zebrafish (*Danio rerio)* use as animal models has gained significant momentum in the recent years due to the multitude of advantages it brings compared to other animal models, such as reduces costs, fast life cycle, the rich repertoire of genetic modifications they can be subjected to and *ex-utero* fertilization and embryonic development. Moreover, more than 70% of human genes have at least one ortholog in zebrafish (Spence et al., 2008; Vaz et al., 2019). Clinical studies, as well as research based on the use of this animal model can illustrate important etiology and pathological traits associated with psychiatric disorders (Kalueff et al., 2014; Meshalkina et al., 2018). Despite the popular opinion that less complex animal exhibit less complex characteristics, previous studies showed that zebrafish` behavior covers most of the cognitive and socio-affective traits in the same way or really similar to human behavior (Ashwin et al., 2014).

Zebrafish exhibit a large spectrum of responses to visual, kinetic and olfactive stimulus and are capable of learning and expressing different types of memory and complex behavior such as anxiety/fear, cognition, social behavior, reward-based behavior, pain-based behavior, and sleep (Kalueff et al., 2013; López-Schier, 2019).

All in one, zebrafish are a good candidate for the study of stress response mechanisms, brain functions, behavior and targeted drug discovery for disorders that affect the central nervous system (Kalueff et al., 2014; Meshalkina et al., 2018).

One of the widely accepted methods to induce schizophrenia-like symptoms in animal models is the administration of sub-anesthetic doses of ketamine in order to mimic the positive symptoms resulted from its impact on the NMDA receptor (Zorumski et al., 2016). Numerous studies have shown that ketamine is a noncompetitive antagonist of the NMDAR and acts by encouraging the channel to open just to block it by binding to a binding situs located deep into the ionic channel and therefore blocks the passing of the ions and remains there even after the channel closes (Zorumski & Izumi, 2012). The channel blocking leads to decreasing of the opening frequency and later on to the inhibition of the inhibitory interneurons which leads to an increased excitation (Sattar et al., 2018). Studies suggest that ketamine aggravates the symptoms of acute stress disorder with strong symptoms of dissociation, flashbacks and avoidant behavior as during exposure to stress, glutamate is released in the cortico-limbic system and may lead to neurotoxicity and behavioral changes with transient dissociation (Rutkofsky et al., 2017).

However, studies on humans have shown that an essential amino acid, namely methionine, might play a role in the psychiatric manifestation as well. Generally, methionine is involved in the homeostasis of multiple systems, as well as its involvement in growth, development and immune response, but it is also one of the most toxic amino acids (Zanandrea et al., 2020). Moreover, studies have highlighted that administrating methionine to schizophrenia patients actually worsened the psychotic symptoms, whereas in rats it induced negative symptoms such as social isolation, increased aggressivity or reduced time spent with conspecifics (L. Wang et al., 2016; Chen et al., 2021). However, most studies regarding its effects are conducted in rats, therefore for the present paper we wanted to verify if the results can be replicated and if there are easier methods to model this disorder, more specifically, isolation. The reason behind also integrating social isolation into the modelling of this disorder is due to the fact that the studies have shown that social isolation can lead to glucocorticoids and oxytocin release, dopamine, serotonin and GABA dysfunction as well as NMDAR sensitivity alteration (Mumtaz et al., 2018).

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Therefore, the fact that ketamine seems to mimic positive symptoms (hallucinations, delusions), whereas methionine seems to mimic de negative symptoms (cognitive and social deficits) make these elements excellent study candidates both administered by themselves and in combination.
The purpose of the paper was to analyze potential new methodologies of modeling schizophrenia that better represent the symptomatology profile observed in humans in order to improve the understanding and clinical potential for this disorder.

2. MATERIALS AND METHODS

Animals

For the purpose of this study there were 40 fishes used, wild-type, acquired from an authorized local seller and manipulated according to the European and Romanian Legislation on the use of animal models. Zebrafish were kept at 28°C in aquariums with water pumps, 12:12 light: dark cycle and fed once a day with commercially available food. The water parameters were tested and maintained within the following limits: nitrites 0-10 NO_2 mg/L, nitrates 0-2 NO_3 mg/L, hardness 4-7 dGH GH, alkalinity 6-10 KH, pH 6-7.5, chloride 0-0.2 mg/L. All experiments were performed two hours after sunrise. Following acclimatization, the zebrafish were split into 4 main groups (control, ket, met, k+m) with 10 fishes each, and each fish was then placed in its own individual enclosure with covered walls so they could not see each other for a period of 14 days during each they were also treated with ketamine and/or methionine.

Substances administration

Based on the study of Wang et al, 2016 (L. Wang et al., 2016) we chose to use a concentration of 6.0 mM methionine (Sigma-Aldrich, ≥98%, MM=149.21 g/mol) because at this concentration there have been significant behavior changes associated with the cognitive and negative symptoms of schizophrenia. The solution was prepared *de novo* daily in 5 L of water which was used as medium and replaced daily for a period of 7 days.
The exposure to ketamine was for a period of 5 days. Each day, the fish were removed for their appointed enclosure and added to a vessel with a ketamine solution of 0,1% for 5 minutes. Although the literature suggest a concentration of 0,2% (Michelotti et al., 2018) for inducing schizophrenia, in the case of our fish that concentration had an anesthetic effect therefore we halved it. The solution was prepared daily using veterinary ketamine (Institutul Pasteur, Romania, 1 mL injectable solution = 100 mg ketamine chlorhydrate). After the five minutes of exposure, the fish were returned to their dedicated aquariums.
For the co-administration groups, the corresponding fish were exposed to 48h of methionine solution only and starting with the third day, they were also exposed to ketamine.

Behavioral tests

Novel Tank Test – The experimental apparatus consists of a rectangular aquarium (20x30x20 cm) filled with 6 L of water and equally split in a top half and a bottom half. This test is normally used for assessing anxiety (the normal behavior is staying in the bottom half of the tank) and the parameters followed for a period of 6 minutes were: latency to first (s), time spent in top half (s), no of entries in upper half, total freezing duration and no of rotations.
Aggressivity and social preference tests: The experimental apparatus consists of a T maze, one of the short arms has a mirror (for aggressivity) or a see-through panel (for social preference) with conspecifics places behind it. The zebrafish is released in the long arm and its behavior is recorded and analysed for 5 minutes. For the aggressivity test the parameters of interest were: the number and duration of swim burst (towards the mirror), the time spent in the left arm (the arm with the

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mirror) and the time spent in decision point. Whereas for the social preference test the parameters followed were time spent in decision point (s), latency to first – left arm (s), number of entries left arm and time spent in left arm (s).

Statistical analysis

Th data were collected using EthoVision XT (Noldus). The data were analyzed using one-way ANOVA with Prism GraphPad 9.3.0. (trial version) followed by post-hoc analysis (Tukey's test). Results were considered significant if $p<0.05$) and data were expressed as mean ± SEM.

3. RESULTS AND DISCUSSIONS

It is well known that Novel Tank Test is a reliable tool to assess zebrafish anxiety and the comparison followed by the interpretation of the results is quite straight-forward in the sense that the normal behavior is to stay at the bottom of the tank or exclusively swim in the bottom half.

In terms of latency to first time reaching the upper half, the results can be visualized in Figure 1. The statistical analysis revealed a significant overall difference of the studied group ($p=0.028$; R sq=0.2994), however the post-hoc analysis showed no significant difference between the groups.

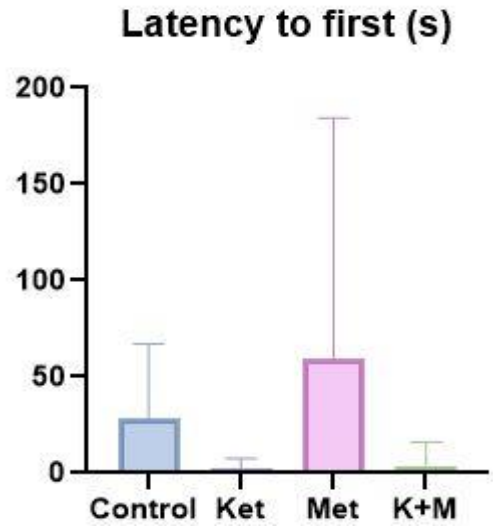

Figure 1. Latency to first entering in the upper half

Although there are no significant differences between groups in terms of latency to first, it can be observed that both the Control and Methionine groups took longer to enter the upper half compared to the Ket and Ket+M groups which supports the literature findings that ketamine reduced anxiety whereas methionine increased it.

Altogether latency to first is not enough to appreciate the anxiety level of the groups as simply entering the upper half fast can only be due to the fish` curiosity or altered spatial orientation when entering the aquarium. Therefore, in order to better assess this aspect, we also analysed the number of entries and the total time spent in the top half.

In terms of the time spent in upper half (s), the ANOVA test revealed significant overall differences ($p<0.001$), whereas the post-hoc analysis showed significant differences between Control and Ket ($p=0.006$), Control and K+M ($p=0.0007$), Ket and Met ($p=0.0045$), and between Met and K+M ($p<0.0001$). The results are graphically illustrated in Figure 2.

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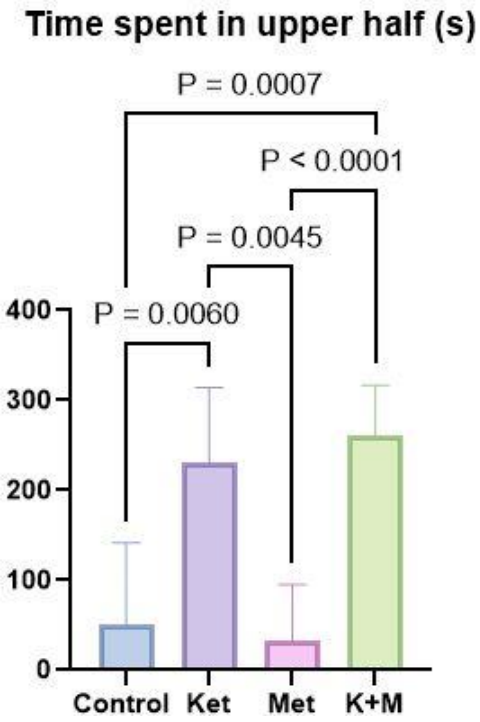

Figure 2. Total time spent in upper half (s) with significant differences between groups

Whereas in terms of the number of entries in upper half, we have identified a significant relationship ($p<0.0001$) with significant differences between Control and Ket ($p=0.0068$), Control and K+M ($p=0.0027$), Ket and Met ($p=0.0069$) and Met and K+M ($p=0.0027$) as can be observed in **Figure 3**.

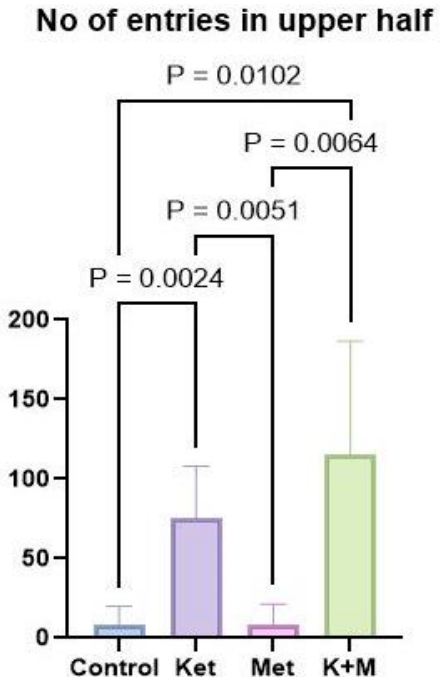

Figure 3. Total number of entries in the upper half with significant differences between groups

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Another parameter frequently used for assessing anxiety in zebrafish is the total duration of freezing episodes. We defined freezing episodes as periods longer than 1s with no movement. The analysis no significant relationship (p=0.7493) and no significant differences amongst groups (p>0.05) as can be observed in **Figure 4.** Although we identified no significant differences between groups in terms of total freezing duration, it can be observed that the Ket and K+M groups had shorter periods compared to the control and K+M groups.

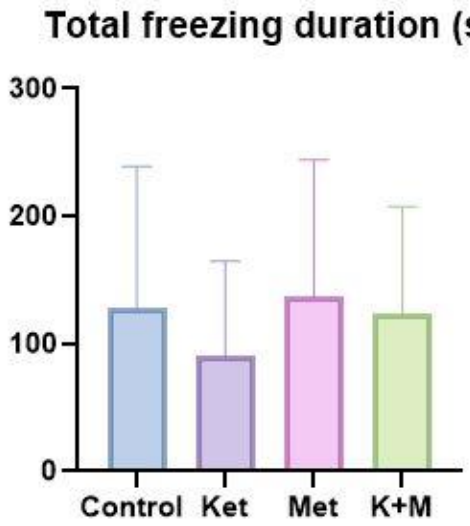

Figure 4. Total duration of freezing episodes with no significant differences between groups

The last parameter of interest in terms of anxiety is the circling behavior manifested through clockwise and counterclockwise rotations. For this purpose, we assessed the number of rotations in both directions. There are no significant differences amongst groups as can be observed in **Figure 5**. However, in the case of clockwise rotations, the number of rotations progressively increased with each group, whereas in the case of counterclockwise rotations the control and Met groups did more rotations compared to the Ket and K+M groups.

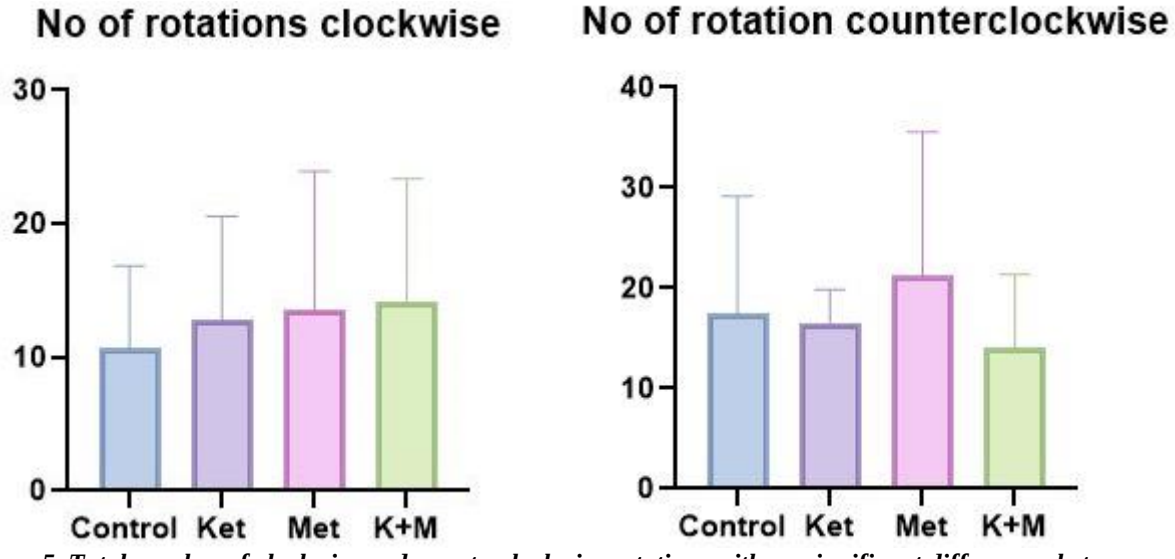

Figure 5. Total number of clockwise and counterclockwise rotations with no significant differences between groups

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Next parameter of interest in modelling schizophrenia is assessing the aggressivity levels through the T-maze test. The first parameter measured was the time spend in left arm (s) (the arm with the mirror) with no significant relationship (p=0.1054) and no significant differences amongst groups (p>0.05) as it can be observed in **Figure 6.** As can be observed, the Control and Met groups spent more time close to the mirror compared to the Ket and K+M groups.

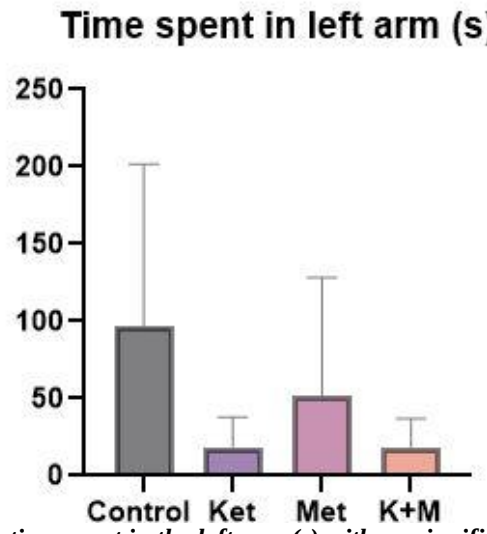

Figure 6. Total duration of the time spent in the left arm (s) with no significant differences between groups.

One important parameter in terms of aggressivity for zebrafish is the swim burst. Swim bursts represent movement at high-speed towards a direction usually followed by an escape type of behavior. We identified no significant relationship or differences between groups in terms of the number of swim bursts and their total duration. However, the number of swim bursts gradually decreased with each group whereas the total duration of swim bursts was higher for all treated groups compared to the control as can be observed in **Figure 7**.

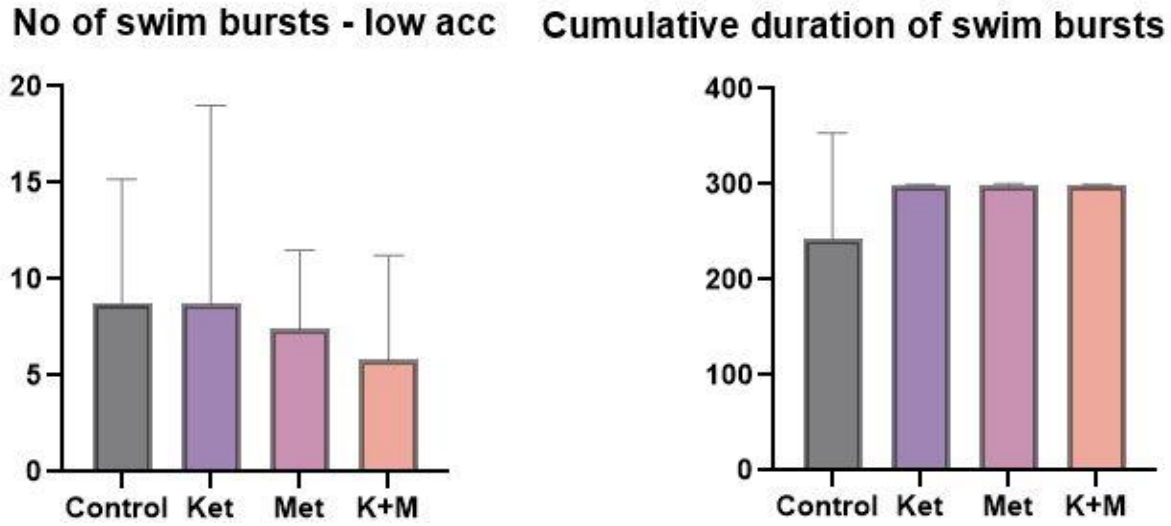

Figure 7. The number and total duration of swim bursts with no significant differences between groups

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Lastly, the social preferences test relies of zebrafish natural behavior to shoal expressed through choosing to spend time close to conspecifics rather than alone. The reasoning behind choosing to perform this test is due to the fact that in schizophrenia there are social impairments and avoidance behavior in terms of social interactions.
For this purpose, we analysed the latency to first left arm (the arm with the conspecifics separated by a see-through screen), the number of entries and the time spent in the left arm.
In terms of latency to first left arm, we identified no significant relationship and no significant differences amongst groups. However, the control and K+M groups took less time than the Ket and Met groups to go to their conspecifics as can be observed in **Figure 8**.

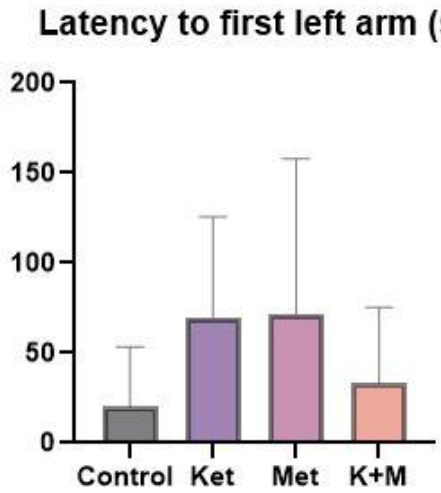

Figure 8. Latency to first entry in the left arm (the arm with conspecifics) with no significant differences

This measurement was accompanied by the total number of entries in the left arm and the time spent in the left arm (s). For both parameters, we have identified no significant relationship or differences amongst groups. The number of entries has been reduced for all groups, but the Met group had the lowest number of entries. Whereas, in terms of the time spent in the left arm, all groups spent a low amount of time (~50 seconds out of 300s) near conspecifics and the Ket and K+M groups spent even less time compared to the Control and K+M groups as can be observed in **Figure 9**.

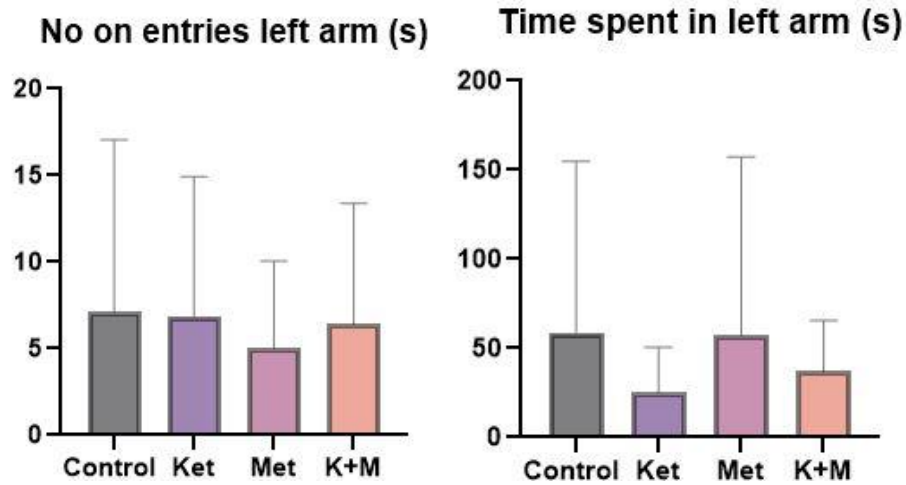

Figure 8. No of entries and time spent in the left arm (the arm with conspecifics) with no significant differences

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Animal models hold a significant importance in clinical research through the advantages they bring in terms of investigating the etiology and potential treatment for a specific disorder or disease. However, the global initiative to reduce the use and suffering of animal models requires the discovery and development of more lenient and accurate modelling of disorders. For this purpose, in the present paper we investigated whether social isolation might be enough to portray schizophrenia behavioral changes against well-established models (ketamine and methionine) and whether a combination of the 2 established substances might give a more accurate depiction of schizophrenia. The social isolation idea is a continuation of a previously published study obtained through a collaboration of our study group with a research group from Egypt that analysed the potential of using social isolation to portray schizophrenia in male Albino rats (Estaphan et al., 2021).

Studies have shown that sub-anesthetic administration of ketamine significantly alters the time spent in top half, number of entries and latency to top (Riehl et al., 2011), whereas in the case of methionine, a 6 mM determines substantial increase in movement and exploration in the upper half compared to a non-treated control (L. Wang et al., 2016) which are in accordance to our results and validate them. In regard to, our new potential models, the isolated control group spent a little more time than the Met group in the upper half but significantly less than the other 2 groups, whereas the co-administration group spent significantly more time in the upper half compared to the Met and control group. Results are similar in terms of the number of entries and latency to first. These results suggest that social isolation alone might actually be mimicking the negative symptoms of schizophrenia as it showed higher anxiety, whereas the K+M group exhibited the highest neurotoxicity and significantly more pronounced behavioral changes out of all the study groups.

In terms of aggressivity behavior, one study suggest that the administration of ketamine in rats determined decreased aggressive behavior (Becker & Grecksch, 2004), whereas in zebrafish it increased it (Michelotti et al., 2018). In the case of our study, the control group spent the most time close to the mirror followed by the Met group, while the Ket and K+M groups avoided the area. This supports the idea that both Ket and Met decreases aggressivity whereas social isolation increases it (Shams et al., 2018).

Surprisingly, literature findings suggest that ketamine administration whether chronic or acute in zebrafish increases social preference, manifested especially through increased time in the social zone and social interactions (Benvenutti et al., 2022), whereas methionine induces social impairments in rats (Lien Wang et al., 2015) and in zebrafish (L. Wang et al., 2016). However, in the case of our study ketamine did not increase social interaction as the Ket and K+M groups had the lowest time spent near the conspecifics and the longest latency to first entry. Interestingly, the methionine and the control group spent similar time near the conspecifics and more than the other 2 groups which suggest that social isolation alone might induce the same type of social impairments as methionine, however the control group took the shortest amount of time to enter the left arm for the first time.

4. CONCLUSIONS

In conclusion, the results illustrate a potential of new schizophrenia models which would be more merciful or accurate compared to the well-established existing models. This idea is supported by the fact that social isolation alone determined behavioral changes similar to the pharmaceutical treated groups, especially compared to methionine. Taking into account the high toxicity of methionine, replacing it with just social isolation in order to mimic the negative symptoms might be a more

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beneficial method. Moreover, the fact that there are no significant differences in most parameters could be due to the social isolation which further suggests that isolation alone could be a potential method to mimic schizophrenia-like symptoms. The limitations of the study are regarding the absence of not isolated groups to further compare the validity of the models; therefore, a future study would focus on obtaining the animal models without the social isolation in order to better assess the potential of the models suggested here.

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