Neurotoxicity Evaluation of Rutin Trihydrate vs. Metformin in Zebrafish Larvae: A Comparative Risk Assessment Study

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ABSTRACT

To examine the neurotoxicity induced by the novel drug Rutin trihydrate and metformin in the zebrafish larvae model. Test solutions for the exposure groups were prepared by diluting the stock solution with egg water. These solutions contained 30 µM metformin in combination with 9.5 µM of the innovative drug rutin trihydrate.

Maintenance of zebrafish and egg collection: Breeding groups were chambered in a specific spawning tank in a male-to-female ratio of 1:1. The spawning tank is provided with a box and with mesh as the bottom base to collect the zebrafish embryos and to protect the zebrafish embryos from the adult fish.

Embryotoxicity assay: This work includes 2 groups and a total sample size of 30. Group 1 underwent examination using the novel drug Rutin trihydrate, which exhibits solubility in 0.01% of Dimethyl sulfoxide (DMSO), while Group 2 was subjected to examination using metformin, soluble in 100µl of DMSO (Dimethyl sulfoxide) combined with 99% water, both assessments conducted on larval zebrafish. Embryos were segregated for each exposure in groups in petri plates with n= 15 embryos per plate and were exposed to the novel drug Rutin trihydrate from 4-96 hpf (Hours Post Fertilization). Other conditions required for the validation of this study were maintained as stated by the OECD.

All the experiments were carried out in triplicate. Statistical analysis: SPSS software facilitated the determination of statistical significance between the two groups. The parameters adhered to a confidential ratio of 95%, a threshold of 0.05, G power at 80%, and an enrolment ratio of 1. Results indicated a significant neuroprotective effect of rutin trihydrate-treated larvae (27.77±1.15) compared to metformin-treated larvae (18.0±0.68), displaying a statistical significance of p = 0.000 (p < 0.05).

Keywords: Toxicity, Embryo, Novel Drug Rutin Trihydrate, Metformin, Cognitive Impairment, Diseases.

1 Introduction

A bioflavonoid called Rutin trihydrate is abundantly found in citrus fruits. It is found in the plant species Carpobrotus edulis. Rutin trihydrate is also called rutoside, sophorin, quercetin-3-O-rutinoside. The molecular weight of rutin trihydrate is 664.56 g/mol.
Metformin is an antidiabetic drug, most specifically it is used for cancer and various other syndromes [1]. The study aimed to treat the cognitive impairment induced by reactive oxygen species by treating it with metformin and rutin trihydrate in zebrafish larvae. Even after accounting for coronary heart diseases, age, blood pressure, weight, and cholesterol readings, the elevated risk of heart failure in diabetes individuals is maintained. Regardless of their condition with regard to coronary diseases, women with diabetes proved to be particularly sensitive and experienced congestive heart failure twice as frequently as males [2].

Research substantiates the therapeutic potential of rutin trihydrate, indicating its efficacy in exhibiting anti-inflammatory and anti-apoptotic effects [3-5].

There are few studies on flavonol glycosides' toxicity, and hardly anything is known about its chronic toxicity. Studies on chronic toxicity are particularly crucial because clinical experience suggests that treatment must be continued for extended periods of time to be successful. Rutin, quercitrin, hesperidin, and naringin were given to dogs either orally or parenterally; some of these substances were excreted in the urine unmodified and none were poisonous. According to clinical studies, rutin can be administered orally once daily for up to 9 months without the patients exhibiting any signs of drug toxicity [6]. In metformin, Hemolytic anemia, vitamin B12 insufficiency, and gastrointestinal disturbance are among the side effects of using biguanides therapeutically. Metformin intoxication can result in hyperlactatemia diseases and metabolic acidosis, despite the fact that this is a rare occurrence. Since the kidneys are the organs that remove metformin from the body primarily, toxicity can happen when metformin accumulates because of inadequate renal clearance or when it is taken in excess.

The level of hyperlactatemia and the extent of acidemia haven't demonstrated any predictive capacity, despite being markers of metformin toxicity. Irrespective of the toxicity's origin, the treatment should encompass supportive measures and the assessment of additional therapies such as gastrointestinal cleansing, glucose and insulin administration, alkalinization, and extracorporeal techniques to reduce metformin levels in the body and restore metabolic balance [7].

The assessment of the potential toxicity of the newly developed drug rutin trihydrate involved the measurement of motility at 96 hours post-fertilisation (hpf). The novelty of this study lies in assessing the toxicity stemming from early exposure to the emerging drug rutin trihydrate, both in its environmental prevalence and its human exposure levels. Therefore, this investigation seeks to shed light on the effects triggered by early prenatal or postnatal exposure to this novel drug. Rutin, the novel drug under scrutiny, has demonstrated protective benefits against various diseases and drug-induced toxicities such as heart issues and memory impairments linked to doxorubicin and cisplatin.

Literature pertinent to this study was gathered from databases like Google Scholar and PubMed, yielding approximately 29,700 and 1067 relevant articles, respectively. Among these, [8] led with 107 citations, followed by [9] and [10] each garnering 66 and 67 citations, respectively. Moreover, [11], standing at 107 citations, emerged as the most impactful study in line with my research interests.

Notably, there remains a gap in current research regarding comparative assessments of behavioural toxicity in zebrafish embryos treated with rutin trihydrate. This study aims to explore the potential of rutin trihydrate, in conjunction with metformin, in treating cognitive impairments, an area yet to be thoroughly investigated.

2 Methodology

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samples. To determine the appropriate sample size for each group, we employed an online sample calculator available at clinicalc.com.

The embryos used in our research were divided into different exposure groups within petri plates, with each plate containing n=15 embryos. These embryos were then exposed to rutin trihydrate from the 4th hour post-fertilization (hpf) to the 96th hpf. To ensure the reliability of our results, we conducted each experimental phase three times, maintaining a consistent static exposure environment throughout.

Our source of Rutin Trihydrate [CAS:250249-75-3, purity 99%] was obtained from Sisco Research Laboratories Pvt. Ltd. in Chennai, India. To prepare a 1000 ppm stock solution, we used ethanol and autoclave water as solvents. This stock solution was carefully stored at -20°C in an amber-colored container. For the test solutions used in both exposure groups, we diluted the stock solution in egg water, following the composition specifications outlined in OECD Technical Guideline TG203 Annexure-2. This egg water composition consisted of 294.0 mg/L CaCl₂·2H₂O, 123.3 mg/L MgSO₄·7H₂O, 64.7 mg/L NaHCO₃, and 965.7 mg/L KCl to achieve the desired final concentration.

2.1 Zebrafish Care and the Retrieval of Eggs

Adolescent zebrafish of the wild-type strain were nurtured in a controlled environment with a constant temperature of 26±1 °C, adhering to a light-dark cycle of 14 hours of light followed by 10 hours of darkness. These zebrafish were acquired from a local breeder, ensuring their genetic purity.

To initiate the process of generating embryos, specific breeding pairs were accommodated in a designated spawning tank, with a deliberate effort to maintain an equal male-to-female ratio of 1:1. The bottom of this tank was equipped with a specialized configuration featuring a protective box and mesh, designed to facilitate the collection of embryos while safeguarding them against potential consumption by mature fish. This breeding setup was meticulously maintained within the E10 AHS block of the Saveetha School of Engineering. Upon the induction of spawning, synchronized with the onset of the light cycle and repeated 30 minutes later, the eggs were gently harvested and thoroughly rinsed in dilution water. Subsequently, the eggs were evenly distributed across four separate petri plates to expose them to distinct dosage conditions.

2.2 Preference Test for Behavioral Partition

Zebrafish larvae were subjected to a treatment regimen involving two substances: metformin at a concentration of 30µM and a novel drug known as rutin trihydrate at a concentration of 9.5μM. Subsequently, neurotoxicity was induced by introducing 1mm of hydrogen peroxide (H₂O₂) into the experimental environment.

The experimental setup was carefully designed, featuring an internal glass chamber with a separation distance of 1 cm. This chamber effectively divided the vertical motion plate into two distinct sections while maintaining the water level at 3 cm. Specifically, the left portion of this chamber, referred to as the "target chamber," was contrasted with the right portion known as the "house," as described by Dubey et al. in their 2015 publication.

The assessment of cognitive function was primarily based on a metric known as the "time spent in the target chamber" (TSTC). This measure provided valuable insights into the cognitive abilities of the zebrafish larvae by observing their behavior within the designated target chamber.

2.3 Statistical Analysis

The data presented in our study were expressed as mean values accompanied by their corresponding standard deviations (SD). To conduct the statistical analysis and create graphical representations, we employed SPSS (Statistical Packages for the Social Sciences).
software, version 20.0, specifically designed for Windows, developed by SPSS Inc. in Chicago, IL, USA.

Our study involved independent variables, namely, Metformin and the novel drug rutin trihydrate, with no dependent variables in the analysis. To compare the groups and assess the significance of our findings, we employed a student t-test. In all our assessments, statistical significance was established at a p-value of 0.000 (p < 0.05), as indicated by previous research [12].

3 Results

Table 1 in our study presents a comprehensive set of group statistics, including mean values, standard deviations, and standard error of the mean. We aimed to assess the neuroprotective abilities of two compounds, rutin trihydrate and metformin, using a partition preference test involving zebrafish larvae. Our primary focus was on measuring the time spent in the target chamber (TSTC) by these larvae following exposure to these novel substances.

Remarkably, our results unveiled a significant difference in neuroprotection, with rutin trihydrate (at a concentration of 9.5 µM) exhibiting a substantially enhanced effect (mean of 27.77±1.15) compared to metformin (mean of 18.0±0.68).

Table 2 displays the outcomes of an independent t-test, which compared the two groups. This statistical analysis confirmed a highly significant finding, with a P-value of 0.000 (P<0.05). This establishes the substantial distinction in neuroprotective potential between rutin trihydrate and metformin.

Table 1. TSTC (Time Spent in Target Chamber) exhibited by zebrafish larvae upon administration of rutin trihydrate and metformin[13].

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>STD. deviation S</th>
<th>STD. error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>15</td>
<td>18.000</td>
<td>.68139</td>
<td>.17593</td>
</tr>
<tr>
<td>Rutin Trihydrate</td>
<td>15</td>
<td>27.767</td>
<td>1.14746</td>
<td>.29627</td>
</tr>
</tbody>
</table>

Table 2. TSTC Equal Variances, assumed for both Levene’s Test for Equality of Variances and the T-test for Equality of Means.

<table>
<thead>
<tr>
<th>Group</th>
<th>Levene’s test for equality of variances</th>
<th>T-test for equality of means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>SIG</td>
</tr>
<tr>
<td>TSTC</td>
<td>5.164</td>
<td>.031</td>
</tr>
</tbody>
</table>

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caused by H₂O₂, notably brought about by the novel drug rutin trihydrate (9.5µm), as

![Graph showing the assessment of time spent in the target chamber (TSTC) between metformin and rutin trihydrate](image)

**Fig. 1.** The time spent on the target chamber (TSTC) was calculated in zebrafish larvae exposed to the novel drug Rutin trihydrate and metformin. The error bars show +/- 1 SD.

### 4 Discussion

Rutin trihydrate possesses therapeutic attributes, exhibiting anti-inflammatory and anti-apoptotic effects. Assessing the neuroprotective potential of rutin trihydrate and metformin, a novel drug, involved a partition preference test in zebrafish larvae. In comparison to metformin, rutin trihydrate showcased a 9% increase in its neuroprotective effect against H₂O₂, demonstrating statistical significance (p<0.05) with a calculated significance level of p = 0.001. This establishes the superiority of rutin trihydrate over metformin in this context.

In line with our findings, behavioural toxicity assessments in fish experiencing cognitive impairment can employ two techniques: the partition preference test and the horizontal compartment test. The objective chamber, placed where fish access food without disturbance, allows assessment of the time spent by zebrafish affected by cognitive impairment, triggered by reactive oxygen species. Metformin administration in zebrafish might lead to lethal consequences, including scoliosis, tail and craniofacial malformations, and yolk deformation.

Flavonoids like rutin exhibit diverse pharmacological effects due to their inherent nature. Given the higher cardiovascular risk profiles in type 2 diabetic patients, metformin's role in enhancing insulin sensitivity has led to its increased use as a primary medication in clinical settings. The future direction of interventions and the effective strategies should be known to healthcare professionals and policymakers, considering the significant investments made in developing and evaluating interventions aimed at improving diabetes care for the public.

Metformin usage has recently gained approval for type 2 diabetic patients, and its future indications might extend to insulin-resistant individuals at high risk of developing type 2 diabetes.
diabetes or exhibiting symptoms of insulin resistance syndrome.

However, limitations exist within the current study, focusing solely on cognitive impairment induced by reactive oxygen species in zebrafish larvae. While rutin demonstrated smooth muscle relaxant properties in vitro, in vivo experiments showed no effect on blood pressure or the respiratory system in guinea pigs. To comprehend the complete potential of drugs in treating cognitive impairment, employing higher model organisms like rats and conducting diverse decision-making tests, such as T-maze, represent a future avenue for exploration.

5 Conclusion

Ongoing findings reveal the significant impact of early developmental exposure to rutin trihydrate, a novel drug at a concentration of 9.5 µm, on zebrafish. This exposure notably mitigated cognitive impairment induced by reactive oxygen species compared to metformin at a concentration of 30 µM. Zebrafish subjected to rutin trihydrate exhibited a 9% increase in chamber dwelling time compared to those treated with metformin, indicating the potential therapeutic use of rutin trihydrate in addressing neural issues. Moreover, the study underscores the correlation between substances causing neurotoxicity in humans and their similar effects in zebrafish. Notably, various substances such as 6-hydroxydopamine, acrylamide, taxol, neomycin, TCDD, retinoic acid, and ethanol induced diverse neurotoxic effects in zebrafish, ranging from neuronal oxidation, demyelination, neuronal apoptosis to defects in optic nerves, motor neurons, and neuronal proliferation.

References


