The assessment of \textit{in vitro} cardiotoxic potentials for synthetic cannabinoid, AM-2201

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\textbf{ABSTRACT}

Synthetic cannabinoid abuse has become more common in recent years, although knowledge about the risk of the relatively new synthetic cannabinoid molecules is not adequate. Data is limited and analytical methods and case reports related to the clinical effects of this substance are recent and new. The studies are generally related to the cardiac effects of first defined molecules rather than every molecule in the group. The cardiac clinical effects of synthetic cannabinoid abuse and its underlying mechanisms are not certain. In this regard, this study aims to investigate AM-2201, one of synthetic cannabinoids, because knowledge related to AM-2201 is less than the others in this group. The cardiotoxicity and underlying mechanisms of AM-2201 were assessed on cardiac cell culture. The half-maximal inhibition concentration ($IC_{50}$) values were 101.49 and 63.33 $\mu$M by WST-1 and LDH assays. AM-2201 did not induced the reactive oxygen species (ROS) levels. Correlatively, no change was observed in total antioxidant capacity (TAC) levels. As to the measurements, Annexin V-FITC and acridine orange dye, AM-2201 did not induce apoptosis and the primary cell death was necrosis. According to our results, further studies such as mechanism on cell death and cancer pathways should be investigated.

\textbf{Keywords:} AM-2201, apoptosis, cardiotoxicity, cytotoxicity, oxidative stress, synthetic cannabinoid

\textbf{INTRODUCTION}

Synthetic cannabinoids elicit cannabimimetic effects similar to $\Delta 9$-tetrahydrocannabinol ($\Delta 9$-THC) which is the primary psychoactive component of cannabis through interaction with CB1 and CB2 cannabinoid receptors. Because synthetic cannabinoids are full agonist and can bind to the CB1 receptor with an affinity greater than $\Delta 9$-THC, they are more potent and have a longer effect than $\Delta 9$-THC. So, they are associated with more severe and dangerous health effects than marijuana (McKeever et al. 2015; Castellanos and Gralnik 2016).

Synthetic cannabinoids, which are considerably new molecules, were initially developed for research purposes as potential therapeutics; however, their abuse was firstly reported and gradually increased in the early 2000s (Debruyne and Boisselier 2015; Cooper 2016; Hess et al. 2016). This increase in synthetic cannabinoids abuse is attributed to intense psychoactive effects and lack of detectability in routine drug screening tests. Despite the molecules being synthetic and consisting of unknown mixtures of chemicals (mostly more than one type of synthetic cannabinoid or other drugs), users believe that the products are natural and harmless (Castaneto 2014; Ibrahim and Al-Saffar 2014; McKeever et al. 2015; Castellanos and Gralnik 2016). In synthetic cannabinoid abuse, agitation or irritability, anxiety, confusion, psychosis, nausea and vomiting, shortness of breath, tremor, and seizures have been mostly reported (Castaneto 2014; McKeever et al. 2015). Cardiovascular side effects such as tachycardia, hypertension, chest pain, and myocardial infarction have been reported in case reports (Aksel 2015; Atik et al. 2015). Most studies in this area have been...
about the effects of synthetic cannabinoids on the nervous system, whereas, knowledge about the toxic effects of synthetic cannabinoids on the cardiovascular system is limited. This study aims to define the toxic potentials and underlying mechanisms of AM-2201, synthetic cannabinoid, on cardiac cells.

**MATERIALS AND METHODS**

**Cell culture conditions:** The rat cardiomyoblast cell line, H9c2 (CRL1448™), was obtained from the American Type Culture Collection (ATCC), and cultured according to the manufacturer’s instructions. AM-2201 was obtained from Lipomed (W-1503-1, Arlesheim-Switzerland). AM-2201 (1 mg) was dissolved in DMSO (27 µL) to prepare the stock solution (100 mM).

For all tests, the cells were seeded in 6-well or 96-well plates at a density of 2.5 x 10⁴ cells/mL, and cultured in 37°C for 24 h. After pre-incubation, each well was treated with 0-100 µM AM-2201 for 48 h. The assays and tests were done in triplicate.

**Cytotoxicity:** WST-1 (Roche Cell Proliferation Reagent, Germany) and LDH (Roche, Germany) assays were performed according to the manufacturer’s instructions.

In WST-1 assay, after exposure to AM-2201, 10 µL of the WST-1 solution was added to each well. After incubation for 30 minutes at 37°C, the samples were analyzed using a microplate reader at 420 nm (Biotek, Epoch, Germany). In LDH assay, after exposure to AM-2201, 100 µL/well supernatant was removed and transferred into a clear microplate. To determine the LDH activity in the supernatants, 100 µL reaction mixtures were added to each well, and incubated for 30 minutes protected from light. Then, the absorbance of the samples were measured using microplate reader at 490 nm. The assay medium (200 µL) for background control and Triton-X solution (100 µL) for positive control were added to wells containing 1x10⁴ cells/100 µL/well. The unexposed cells were evaluated as the negative control. To determine cytotoxicity, the absorbance values were measured using a microplate spectrophotometer system (Epoch, Germany). Then, the inhibition of enzyme activity observed in the cells was calculated with the absorbance values and compared to that of unexposed cells. The concentration-cell death (%) curves were used to calculate the half maximal inhibition concentrations (IC₅₀) that are responsible for the death of 50% of the cells. The cytotoxicity was evaluated with IC₅₀ values.

**Oxidative stress:** The induction of ROS production was evaluated by 2,7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Sigma-Aldrich, Germany) dye using flow cytometry (Acea Noocyte 1000, California, USA) at Ex/Em: 488/535 nm (Schupp et al. 2008). The controls and the exposed cells were adjusted to be 1x10⁴ cells/mL. At least 1x10⁴ cells in 6-well plates were analyzed, the results were shown as percent of fluorescence intensity. Phosphate buffer serum (PBS) was used as a negative control. In the results, the presence of ROS was expressed as the percentage of the total cell quantity.

For TAC assay, Total Antioxidant Capacity Assay Kit (Sigma-Aldrich, Germany) was used according to the manufacturer’s instructions. The stock Trolox solution was prepared at 1 mM in DMSO and stored at -20°C. One part of Cu²⁺ reagent was diluted with 49 part of assay diluents for the Cu²⁺ working solution. The working concentrations of Trolox were 4, 8, 12, 16 and 20 nmol/well. After exposure to AM-2201, the Cu²⁺ working solution (100 µL) was added to every well, and incubated for 90 min protected from light. Then, the absorbance was measured at 570 nm by a microplate reader. By using Trolox standard curve, the amounts of Trolox in the samples were calculated as in the following equation:

\[ \text{TAC}_{\text{sample}} = \frac{\text{Ts}}{\text{SA}} \]

Ts: sample volume added in the sample wells (µL)
SA: TAC amounts in the sample well calculated from standard curve (nmol)

**Apoptosis:** Annexin V-FITC detection kit (Biolegend, CA, USA) was used according to the manufacturer’s instructions. The fluorescence intensity was measured in FITC and PI channel (at 494/518 nm (Ex/Em) for Annexin V-FITC and 535/617 nm (Ex/Em) for PI) by flow cytometer. Necrotic cell as Annexin V / PI−, living cell as Annexin V / PI+, early stage apoptotic cell as Annexin V / PI−, late apoptotic cells as Annexin V/PI+ expressions appeared. The results were expressed as the percentage of total cell quantity.

**Autophagy:** Acidine orange dye (Sigma-Aldrich, Germany) was used (Mitou et al. 2009; Gözüaçık 2011). After exposure to AM-2201, the wells were washed with PBS (1X), detached with trypsin (0.2%), and then centrifuged. Then, the cells (2.5x10⁴) were suspended in the cell medium, and incubated with acidine orange suspension (2%) in the dark for 15 minutes. Finally, the absorbance value was analyzed at 488/676 nm (Ex/Em) with PerCP filter by flow cytometer. The results were expressed as the percentage of autophagy seen cells to total cell quantity.

**Statistical analysis**

Data were expressed as mean ± standart error of mean (SEM). One-way ANOVA, Post Hoc and Dunnet t-test using SPSS version 23 for Windows (IBM Corp.; Armonk, NY, USA) was used to analyse the data. p<0.05 indicates a statistically significant difference.

**RESULTS AND DISCUSSION**

Despite the increasing use of synthetic cannabinoids, the reported serious adverse effects and limited toxicology data make their abuse an important public health issue. Some case reports have reported a large number of side effects on the different organs and systems. On the cardiovascular system, tachycardia, hypertension, dysrhythmia, chest pain, cardiac ischemia and myocardial infarction are common adverse clinical effects for synthetic cannabinoid abuse. Knowledge about the toxic effects of synthetic cannabinoids on the cardiovascular system is limited. Most of the studies are about the toxic effects of synthetic cannabinoids on the nervous system. Therefore, we aimed to investigate AM-2201, one of synthetic cannabinoids, because knowledge of AM-2201 is less than that of the others in this group. In addition, we evaluated the potential of cytotoxic, oxidative and apoptotic damage of AM-2201 on H9c2 rat cardiomyoblast cell line.
Firstly, WST-1 and LDH assays were performed for the cytotoxic effects of AM-2201. The IC\textsubscript{50} values were 101.49 and 63.33 µM by WST-1 and LDH assays, respectively (Figure 1). The LDH leakage assay was observed more sensitive than WST-1 assay for AM-2201 cytotoxicity in H9c2 cell line. Tomiyama and Funada (2011) reported that CP-47,497, CP-47,497-C8 and CP-55,940 were significantly cytotoxic for 2 h exposure at 30 µM. CP-55,940 caused nuclear fragmentation and condensation at 30 µM concentration. They also reported that their cytotoxic effects were significantly suppressed by the CB1 receptor antagonist AM-251, but not by the CB2 receptor antagonist AM-630. In the other study conducted by Tomiyama and Funada (2014), it was observed that eight synthetic cannabinoids (HU-210, CP-55,940, CP-47,497, CP-47,497-C8, JWH-018, JWH-210, AM-2201, and MAM-2201) induced a significantly cytotoxic effect in a dose-dependent manner on primary mouse neuronal cells. They reported that the relative cytotoxicity levels of AM-2201 were 2.4 and 2.8 at 10 µM and 30 µM AM-2201, respectively, to the control group. In comparison with our results, the primary mouse neuronal cells could be more sensitive than mouse cardiomyoblast cells to AM-2201.

In the present study, the type of cell death was determined by staining with Annexin V-FTIC-PI. At the highest concentration (100 µM), early stages of apoptosis were not observed, and late apoptosis was determined to be maximum 1.17% (data not shown). Total apoptosis results were shown in Table 1. It was observed that the necrosis ratio was maximum 12.76%. At 100 µM exposure, apoptosis and necrosis were increased 3 and 9.73 fold, respectively, compared to the control (p>0.05) (Table 1).

Tomiyama and Funada (2011) reported that the number of Annexin V\textsuperscript{+} cells was significantly increased at 2 h after treatment with CP-55,940. Also, some morphological changes were induced by CP-55,940, which were reflected the necrotic or late apoptotic cells. Couceiro et al. (2016) indicated JWH-018 metabolite, N-(3-hidroksipentil), decreased the cell via necrosis on human kidney and neuroblastoma cell lines. Similarly, we observed necrotic cell death after treatment with AM-2201.

In the present study, the ROS levels were observed to decrease 15.69% at 100 µM compared to control (p>0.05) (Figure 2). The TAC levels also decreased depending on exposure concentration, however it was not statistically significant (p>0.05). The decrease of TAC was ≤32.18% at the exposure concentrations (Figure 3). However, the results should be ignored because a

<table>
<thead>
<tr>
<th>Concentrations (µM)</th>
<th>Apoptosis [mean%± standard error]</th>
<th>Necrosis [mean%± standard error]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.31±0.03</td>
<td>1.03±0.08</td>
</tr>
<tr>
<td>12.5</td>
<td>0.36±0.04</td>
<td>3.94±0.07*</td>
</tr>
<tr>
<td>25</td>
<td>0.48±0.06</td>
<td>8.27±1.12*</td>
</tr>
<tr>
<td>50</td>
<td>0.76±0.08*</td>
<td>7.22±1.05*</td>
</tr>
<tr>
<td>100</td>
<td>0.94±0.08*</td>
<td>8.05±1.11*</td>
</tr>
</tbody>
</table>

*<p>0.05, one-way ANOVA, Post Hoc and Dunnet t-test.

Figure 1. The effects of AM-2201 on H9c2 cell viability by WST-1 and LDH.

Figure 2. The ROS levels in H9c2 cells exposed AM-2201 at 12.5, 25, 50 and 100 mM concentrations.

Figure 3. Total antioxidant capacity of H9c2 cells exposed AM-2201 at 12.5, 25, 50 and 100 mM concentrations.
high cytotoxicity level was observed at these exposure concentrations.

Rajesh et al. (2010) investigated the cell death and signal transduction pathways related to CB1 receptors activated via endocannabinoid anandamide and HU-210 (synthetic agonist) in human corona artery endothelial cells. They reported endocannabinoid anandamide and HU-210 increased ROS levels, and also CB1 receptor antagonist or antioxidants (acetylcysteine) decreased ROS levels. However, Almada et al. (2017) observed that WIN-55,212 decreased 16% of cell viability in human choriocarcinoma and primer culture of placenta cytotrophoblast cells. Also, they reported that WIN 55,212 did not induce ROS formation. Similar to Almada et al. (2017), we observed that AM-2201 did not induce ROS production. However, the results were not statistically significant (p>0.05) even if AM-2201 decreased ROS levels in rat cardiomyoblast cells.

Data about the effects of autophagy induction of synthetic cannabinoids is not available in the literature. In our study, the autophagic effects of AM-2201 were detected with acridine orange dye. In the rat H9c2 cardiomyoblast cells exposed to AM-2201 during 48 h, the autophagy ratio to total cell was determined to be 57.24-72.40%. The induction was ≤1.28 fold compared to control, however, the results were not statistically significant (p>0.05) (Figure 4).

In conclusion, we observed necrotic cell death principally under the concentrations of IC_{50} values (<63.33 µM). Based on our results, it could be concluded that the cardiotoxic effect of AM-2201 might be due to hypertrophic and/or arhythmmogenic effects rather than direct cardiomyoblast cell death effects. Therefore, it is considered that further studies should be carried out in terms of the effects of metabolites, in hypertrophic signal pathways, and cardiac conduction pathways. Besides, knowledge of most synthetic cannabinoid packages includes multiple synthetic cannabinoid types and other psychoactive ingredients. Therefore, the investigation about interactions between synthetic cannabinoids and other drugs need to be researched.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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**REFERENCES**