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Toxicity evaluation of two polyoxotungstates with anti-acetylcholinesterase activity

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ABSTRACT

A toxicity evaluation of two Keggin-type heteropolytungstates, $K_7[Ti_2PW_{10}O_{40}]\cdot 6H_2O$ and $K_6H[SiV_3W_9O_{40}]\cdot 3H_2O$, with different inhibitory potencies toward acetylcholinesterase activity (IC_{50} values of 1.04×10^{-6} and 4.80×10^{-4} mol/L, respectively) was performed. *Wistar albino* rats were orally treated with single doses (5 and 50 mg/kg) of both investigated compounds. The biochemical parameters of renal (serum urea and creatinine) and liver function (direct and total bilirubin, alanine transaminase, and aspartate aminotransferase) were determined after 24 h and 14 days. A histopathological analysis of liver tissue was carried out 14 days after the polyoxotungstate administration. Both applied doses of the investigated compounds did not induce statistically significant alterations of the renal function markers. However, the polyoxotungstate treatment caused an increase in the activities of serum alanine transaminase and aspartate aminotransferase in a time- and concentration-dependent manner, although statistically significant changes in bilirubin concentrations were not observed. Furthermore, the detected hepatotoxic effect was confirmed by histhopathological analysis that suggested some reversible liver tissue damage two weeks after the treatment, especially in the case of $K_6H[SiV_3W_9O_{40}]\cdot 3H_2O$. Accordingly, the toxicity of these two polyoxotungstates with anti-acetylcholinesterase effect cannot be considered as a severe one, but their potential clinical application would require a more complex toxicological study.

1. Introduction

Polyoxometalates (POMs) are negatively charged inorganic cage complexes containing early transition metal ions in high oxidation states (*e.g.* W^{6+}, V^{5+}) surrounded by oxo ligands (Iqbal et al., 2013). Some of these anionic clusters are stable in aqueous solutions at physiological pH values (Geng et al., 2011). *In vitro* as well as *in vivo* studies suggest biological activities of POMs such as anticancer (Wang et al., 2005), antibacterial (Fukuda and Yamase, 1997; Fukuda et al., 1999), antiviral (Shigeta et al., 1996; Inouye et al., 1995; Ikeda et al., 1993; Yamamoto et al., 1992; Shigeta et al., 1997), and antidiabetic (Nomiya et al., 2001; Balici et al., 2015) activities. Their biological mechanisms of action at the molecular level are not well understood. It has been suggested that POMs have an extracellular mode of action affecting various enzymes, mostly located on the plasma membrane (Krstić et al., 2009; Čolović et al., 2011; Lee et al., 2015). However, a toxic action of POMs was confirmed (Rhule et al., 1998), which presents the main limitation for biomedicinal applications.

In a recent study, Iqbal et al. (2013) demonstrated the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities induced by micromolar concentrations of a group of polyoxotungstates. AChE (EC.3.1.1.7) is a membrane-bound serine hydrolase involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine in numerous cholinergic pathways in the central and peripheral nervous systems (Čolović et al., 2013). The reversible inhibition of brain AChE is the major therapeutic target in the treatment of Alzheimer's disease associated with loss of cholinergic neurons in the brain and a decreased level of acetylcholine (Wenk,

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2006; Herholz, 2008; Noetzli and Eap, 2013).

The aim of our study was to evaluate *in vivo* toxicological effects of two polyoxotungstates, $K_7[Ti_2PW_{10}O_{40}]\cdot 6H_2O$ (**K-Ti$_2$PW$_{10}$**) and $K_6H[SiV_3W_9O_{40}]\cdot 3H_2O$ (**K-SiV$_3$W$_9$**), with different anti-AChE activities (IC_{50} values of 1.04×10^{-6} and 4.80×10^{-4} mol/L, respectively) using *Wistar albino* rats as an experimental model. Iqbal et al. (2013) reported that some polyoxotungstates act as potent AChE inhibitors and consequently as promising therapeutic agents for Alzheimer's disease. Earlier studies (Yamase et al., 1996; Fukuda et al., 1999) indicated an antibacterial activity of these two selected compounds against methicillin-resistant *Staphylococcus aureus* strains, which was patented by Yamase and Tajima (1996). Additionally, the di-Ti-substituted Keggin type polyoxotungstate **Ti$_2$PW$_{10}$** has shown inhibiting properties for the replication of several enveloped DNA and RNA viruses (Ikeda et al., 1993; Shigeta et al., 2003). Since *in vivo* toxicity presents the major limitation for the medical application of novel bioactive compounds (Feng et al., 2002), it was considered important to perform a preliminary *in vivo* toxicological study before doing further *in vivo* anticholinesterase investigations. Although the biological activity of POMs has been investigated for many years, reports of relevant toxicological studies are relatively rare. In order to evaluate the POM-induced toxic effects on renal and liver function, some biochemical and histopathological parameters were followed after oral application of the investigated POMs. In addition, two polyoxotungstates with different inhibitory powers to AChE were chosen for the toxicity studies in order to correlate the induced anti-AChE and toxic effects.

2. Material and methods

2.1. Chemicals

All reagents and chemicals employed for the POM synthesis were of high-purity grade and used as purchased without further purification. AChE (specific activity 288 IU/mg solid, 408 IU/mg protein) from electric eel, acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemicals Co. (Germany). Other medium assay chemicals (sodium dodecyl sulfate (SDS), potassium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate ($NaH_2PO_4\cdot 2H_2O$) were from Merck (Germany).

2.2. Synthesis of POMs

Both POMs used in this study were synthesized according to published procedures. $K_7[Ti_2PW_{10}O_{40}]\cdot 6H_2O$ (**K-Ti$_2$PW$_{10}$**) was prepared according to (Domaille and Knoth, 1983) and $K_6H[SiV_3W_9O_{40}]\cdot 3H_2O$ (**K-SiV$_3$W$_9$**) was prepared according to Finke et al. (1986). $Na_{10}[H_2W_{12}O_{42}]\cdot 27H_2O$, $Na_6[TeW_6O_{24}]\cdot 22H_2O$, $K_6[PV_3W_9O_{40}]\cdot 3H_2O$, $(NH_4)_{14}[NaP_5W_{30}O_{110}]\cdot 31H_2O$ and $K_6H_2[TiW_{11}CoO_{40}]\cdot 13H_2O$ were synthesized according to the previously published procedures, respectively, Evans and Rollins (1976), Mauracher et al. (2014), Domaille and Watunya (1986), Preyssler (1970) and Chen and Liu (1997).

2.3. Acetylcholinesterase assay

The AChE activity in the absence (control) and presence of the investigated POMs was determined by a slightly modified Ellman's method (1961). The experiments were performed by *in vitro* exposure of 0.01 IU commercial enzyme to the investigated POMs in 50 mmol/L phosphate buffer pH 8.0 (final volume 0.650 mL). The standard medium assays were preincubated for 20 min at 37 °C in the absence or presence of the desired concentration of the POMs. Stock solutions (0.001 mol/L) of the investigated POMs were prepared daily by dissolving the solid compounds in water. Working solutions were prepared by diluting (with water) the stock solutions to desired concentrations (as needed), shortly before use. Ten microliter acetylthiocholine iodide (0.075 mol/L) was used as the enzyme substrate in combination with 20 μL DTNB (0.01 mol/L in 50 mmol/L phosphate buffer pH 7.0) as a chromogenic reagent. The reaction was started by the addition of acetylthiocholine iodide (final concentration 0.001 mol/L), and allowed to proceed for 5 min at 37 °C until stopped by addition of 65 μL SDS (10%). The yellow product 5-thio-2-nitrobenzoate, released in the reaction of thiocholineiodide (product of enzymatic reaction) and DTNB, was measured at 412 nm using Perkin Elmer Lambda 35 UV–VIS spectrophotometer (Shelton, USA). The AChE activity was expressed as ΔA_{412}/min/mg protein. All experiments were performed in triplicate.

2.4. Toxicity evaluation

2.4.1. Ethics statement

This study was carried out in strict accordance with the Animal Welfare Act of the Republic of Serbia (Official Gazette of the Republic of Serbia No. 41/09), Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes, and the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th ed., USA). The methodology used in our investigation was approved by the Ethical Commission for the Welfare Protection of Experimental Animals (Faculty of Medicine, University of Belgrade, 323-07-04764/2012-05). Additionally, all experiments were conducted in accordance with OECD Guidelines for the Testing of Chemicals (Section 4/Test No. 420: Acute Oral Toxicity - Fixed Dose Procedure), the OECD Principles of Good Laboratory Practice, including the "3Rs" principle (Radenković, 2012) and finally the Handbook on Non-clinical Safety Testing - World Health Organization (WHO, 2002).

2.4.2. Animals

In vivo experiments were performed on male adult *Wistar* rats ($n = 26$) weighing 250–350 g (293 ± 14 g) receiving a standard diet and water *ad libitum*. The weight of the rats was measured before the start of the experiment and was followed each day throughout the course of the experiment.

2.4.3. Experimental design

The rats were distributed into five experimental groups ($n = 6$ for sham group; $n = 5$ per treated group):

- I: Sham group
- II: **K-Ti$_2$PW$_{10}$**, 5 mg/kg
- III: **K-Ti$_2$PW$_{10}$**, 50 mg/kg
- IV: **K-SiV$_3$W$_9$**, 5 mg/kg
- V: **K-SiV$_3$W$_9$**, 50 mg/kg.

The animals in the first group received a single dose of saline (0.9% NaCl) *per os*. The animals in groups II-V received a single dose of each tested compound *per os*. All experimental animals were followed for two weeks.

After 24 h of POM exposure (1), blood samples were taken from the tail vein of sham and all treated groups (I, II1, III1, IV1) except the V1 group (tail veins were collapsed). At the end of the experiment, after two weeks (2), blood samples were collected *via* cardiac puncture (I, II2, III2, IV2, V2). The samples of collected blood were centrifuged, and the biochemical markers of renal (serum urea – S_{Ur} and serum creatinine – S_{Cre}) and liver function (direct and total bilirubin, aspartate aminotransferase – AST, alanine transaminase – ALT) were determined in the isolated serum samples. The liver samples of each animal were taken *post mortem*, for a histological evaluation.

Since the chosen polyoxotungstates had never been *in vivo* tested, the selection of doses and times for parameters monitoring was made according to recommended protocol (OECD Guidelines for the Testing of Chemicals, Acute Oral Toxicity – Fixed Dose Procedure).

2.4.4. Histological evaluation

2.4.4.1. Tissues preparation. All tissue samples were fixed in 4%