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Ascorbic acid as DNase I inhibitor in prevention of male infertility

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ABSTRACT

Apoptotic and/or ROS-induced DNA fragmentation in sperm cells may contribute to the development of male infertility. As the known dietary antioxidant, ascorbic acid prevents ROS production and protects sperm cells from DNA damage. Here, we found that ascorbic acid has the ability to inhibit DNase I, one of the main endonucleases involved in DNA fragmentation during apoptosis. Site Finder and Molecular docking defined the ascorbic acid interactions with the most important residues of DNase I, including H-donor interactions with Asp 168 and Asn 170, and H-acceptor interaction with Asn 170. As a furan derivative, ascorbic acid could be considered a pioneer of substrate-based DNase I inhibitors. The results indicate to another possible mechanism for prevention of male infertility by ascorbic acid.

1. Introduction

Sperm DNA integrity is essential for the maintenance of genetic health, as well as for the accurate transmission of genetic information [1,2]. Impaired integrity of sperm cells DNA has been observed among infertile patients [3–5]. Reactive oxygen species (ROS) are considered one of the main sources of DNA fragmentation in human spermatozoa [1,6]. However, the administration of oral antioxidants may afford protection to sperm DNA. It was shown that ascorbic acid, a known oral antioxidant, can be efficiently used in the treatment of sperm DNA damage [1,6–8].

Apoptosis (or programmed cell death) is necessary for normal spermatogenesis in mammals and maintenance of cellular homeostasis, whereby a certain number of germ cells are eliminated in order to maintain a precise germ cell population. Increased apoptosis is believed to be one of the main factors contributing to male infertility [9]. Gorczyca et al. [10] showed that the activation of an endonuclease may represent a ubiquitous mechanism of both somatic or germ cell death. Normally, the architecture of spermatozoa is so compact that it prevents any apoptotic nuclease to access the nuclear DNA and induce its fragmentation [11,12]. However, the DNA protection is poorer in men with abnormal semen parameters [11]. Deoxyribonuclease I (DNase I), a Ca^{2+}/Mg^{2+}-dependent endonuclease, is considered one of the main nucleases involved in DNA fragmentation during apoptosis [13–18]. Investigating DNase I-induced sperm DNA fragmentation among different mammalian species *in vitro*, it was shown that human spermatozoa were the most sensitive to DNase I which caused the digestion of the human sperm DNA [19]. Distribution of DNase I in rat testis and its correlation with apoptosis was also described by Stephan et al. [20]. DNase I inhibitors could be therefore useful in the prevention of sperm DNA fragmentation and might be effective in the treatment of male infertility. In order to evaluate another possible mechanism for the prevention of sperm DNA fragmentation, we investigated the ability of ascorbic acid to inhibit DNase I.

Site Finder and Molecular docking are *in silico* drug design methods used to better understand the drug-target interactions that hypothesize the designing of novel drug candidates [21,22]. As a result of missing XRD/NMR atomic-level details between small organic inhibitor and bovine pancreatic DNase I [23], the Site Finder was employed to calculate possible active sites in DNase I. In addition, we wanted to clarify the DNase I inhibitory property of ascorbic acid at the molecular level using the molecular docking.

2. Materials and methods

2.1. Evaluation of DNase I inhibition

DNase I from bovine pancreas, DNA and perchloric acid were purchased from Sigma–Aldrich. Ascorbic acid was purchased from Merck and crystal violet was purchased from Lach-Ner.

DNase I from bovine pancreas was employed for *in vitro*

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evaluation of enzyme inhibition, by spectrophotometric measurement of acid-soluble nucleotides formation at 260 nm (method slightly modified by Bartholeyns et al. [24]). The inhibition was studied in a series of test-tubes with the reaction mixture (total volume of 1040 μL), prepared in a following order: (*i*) *test samples* contained 80 Kunitz units of DNase I, ascorbic acid dissolved in water, 0.0077% of DNA, and 80.77 mM TRIS-HCl buffer (pH 7.6); (*ii*) *solvent control samples* contained the same amount of DNase I, appropriate amount of water, DNA and TRIS-HCl buffer. Corresponding blank samples were prepared for each group in the same way as the test solutions (*i* and *ii*).

After incubation at 37 °C for 30 min the reaction was stopped by adding 80 μL of perchloric acid. The percentage of enzyme inhibition was determined by measuring the absorbance difference that correlates with acid-soluble nucleotides formation; it was calculated as a percentage of specimen absorbance vs. absorbance of the solvent control samples. IC_{50} curve was generated using six concentrations of ascorbic acid (600, 500, 400, 300, 200 and 100 μM). Crystal violet was used as positive control. All experiments were performed in triplicate and averaged.

2.2. In silico studies

2.2.1. Ligand preparation

Ascorbic acid was built with ChemBioDraw Ultra 13.0 (PerkinElmer, Inc.) and molecular geometry has been optimized with ChemBio 3D Ultra 13.0 (PerkinElmer, Inc.) using MM2 force field until a minimum 0.100 Root Mean Square (RMS) gradient was reached. Compound structure was followed by energy minimization with MMFF94x force field in the Molecular Operating Environment (MOE) Software package 2014.0901 [21]. Conformational analysis was carried out by MOE LowModelMD method which performs molecular dynamic perturbations along with low frequency vibrational modes with energy window of 7 kcal/mol, and conformational limits of 1000.

2.2.2. Receptor preparation

The X-ray crystallographic structure of a complex between DNase I and the self-complementary octamer duplex d(GGTATACC)$_2$ (PDB code: 1DNK) was obtained from the Protein Data Bank [25]. The errors of DNase I were corrected by the Structure Preparation process in MOE. After the correction, hydrogens were added and partial charges (Gasteiger methodology) were calculated. Energy minimization (AMBER12:EHT, RMS gradient: 0.100) was performed.

2.2.3. Binding site selection

The Site Finder module of the MOE was used to identify possible ascorbic acid-binding sites within the optimized structure of DNase I. Hydrophobic or hydrophilic alpha spheres of DNase I, served as probes denoting zones of tight atom packing. These alpha spheres were utilized to define and rank potential binding sites according to their propensity for ligand binding (PLB) score, which was based on the amino acid composition of the pocket [26].

2.2.4. Docking protocol

The molecular docking study was performed using MOE to understand the ascorbic acid-DNase I interactions in detail. The default Triangle Matcher placement method was used for the induced fit docking. GBVI/WSA dG scoring function which estimates the free energy of binding of the ligand from a given pose was used to rank the final poses. Ascorbic acid-DNase I complex with lowest relative binding free energy (ΔG) score was selected.

3. Results and discussion

In order to expand the investigations on the role of ascorbic acid in DNA protection, we evaluated its activity against DNase I. As a result, ascorbic acid inhibited DNase I with IC_{50} value of 330.74 ± 29.92 μM. However, it showed to be weaker DNase I inhibitor compared to crystal violet (IC_{50} = 227.34 ± 35.55 μM), used as positive control. Despite its high IC_{50} value, ascorbic acid, either from food sources or supplements, in the recommended dosages, could be useful in the protection of DNA.

The Recommended Dietary Allowances (RDA) for ascorbic acid for adult females and males are set to be 75 and 90 mg/day, respectively. Because of increased oxidative stress and metabolic turnover of ascorbic acid, smokers require 35 mg/day more ascorbic acid than nonsmokers. The tolerable upper intake level for adults for ascorbic acid is set at 2000 mg/day [27]. Greco et al. [8] showed that ascorbic acid reduces the incidence of sperm DNA fragmentation when given at a daily dose of 1 g (500 mg twice a day) for 2 months.

The binding site residues in DNase I have been identified using the Site Finder implemented in the Molecular Operating Environment (MOE) software [21]. The results from the analysis highlighted that amino acid residues like Asn 7, Arg 9, Glu 39, Tyr 76, Glu 78, Arg 111, His 134, Ala 136, Pro 137, Asp 168, Asn 170, Thr 203, Thr 205, Thr 207, Tyr 211, Asp 251 and His 252 constituted the binding site of the DNase I structure (Table 1). Our results are consistent with a recent study highlighting the conservation of the amino acids involved in the identified cation-binding sites across DNase I and DNase I-like protein [23]. It is worth mentioning that ascorbic acid-binding site, represented by a grey-red surface map, is within the region that interacts with DNA octamer d(GGTATACC)$_2$ (Fig. 1A).

The intermolecular contacts between ascorbic acid and DNase I was analyzed using the ligand interaction diagram of MOE suite (Fig. 1B and C). It illustrates the existence of hydrogen bond interactions with Asp 168 and Asn 170 residues (Table 2). Additionally, the bond distances, bond energy and binding free energy between ascorbic acid and receptor atoms were also examined (Table 2). Weston et al. [25] showed that scissile phosphate group of

Table 1
Summary of the possible ascorbic acid-binding sites in DNase I.

Site	Size	PLB	Hyd	Side	Residues
1	50	2.55	13	54	Asn 7, Arg 9, Glu 39, Tyr 76, Glu 78, Arg 111, His 134, Ala 136, Pro 137, Asp 168, Asn 170, Thr 203, Thr 205, Thr 207, Tyr 211, Asp 251, His 252
2	30	0.39	12	23	Val 40, Arg 41, Asp 42, Ser 43, Pro 70, Ser 75, Tyr 76, Lys 77, Glu 78, Arg 79
3	10	0.15	13	16	Val 125, Lys 126, Glu 127, His 159, Leu 160, Asn 161, Asp 162, Leu 220
4	21	−0.05	6	8	Pro 137, Ser 138, Asp 139, Ala 140, Val 141, Ser 174, Tyr 175, Gln 180
5	12	−0.06	8	14	Val 66, Val 67, Ser 68, Phe 82, Thr 94, Tyr 95, Gln 96, Ala 114, Val 115, Val 116
6	5	−0.59	4	11	Arg 9, Thr 10, Lys 15, Leu 247, Ala 248, Ile 249, Ser 250, Asp 251
7	12	−0.67	5	11	Tyr 97, Asp 149, Val 150, Leu 152, Asp 153, Gln 156
8	5	−0.72	7	11	Ala 200, Asp 201, Pro 232, Asp 234, Tyr 253
9	5	−1.00	5	13	Leu 1, Lys 2, Asp 33, Lys 88, Ser 122

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