**INVENTION DISCLOSURE FORM**

Thymoquinone (TQ) is one of the most active constituents of *Nigella sativa* (*N. sativa*), widely being used as a hepato-protective agent. However, toxicity and poor water solubility at higher dosages limit its use as a therapeutic agent. The idea behind the present study is to design a nanocarrier that exploits the benefit of the antioxidant property of TQ without causing any toxicity. For this purpose, PAG (p-aminophenyl-1-thio-b-D-galactopyranoside) coated NIPAAM (N-isopropylacrylamide) nanoparticles were synthesized followed by encapsulation of TQ (Nanothymoquinone, NTQ) in their hydrophobic core. PAG is a ligand that directly interacts with asialoglycoprotein receptors (ASGP-R) present on the surface of hepatocytes and delivers the drug directly to the liver. NTQs were found to have a size of ~90 to 108 nm and were characterized using DLS and TEM. The drug was given in two modes: one as NTQ (3 groups: 0.125 µgkg-1 body weight (NTQL), 1.25 µgkg-1 body weight (NTQM) and 12.5 µgkg-1 body weight (NTQH) µgkg-1, intraperitoneal (i.p.)), and the other as TQ (12.5 mgkg-1 body weight, i.p.).

**EXPERIMENTAL PROCEDURE**

In the present study, we have evaluated the preventive efficacy of TQ and NTQ against CCl4- induced hepato-toxicity (free radical generation). CCl4 administration is a well-known model for the production of chemical hepatic injury, causing an increase in lipid peroxidation and reduction in the activities of the anti-oxidant enzymes such as Glutathione peroxidase (GPX) and Catalase.

Animals were divided into six groups, each having 6 animals and they have received the treatment as follows: Group I (N): Normal (saline for 7 days i.p.), Group II (D): CCl4 treated (CCl4 on 3rd and 4th day, 1.2 ml/kg , subcutaneous (s.c.)) + diet/water, Group III (NTQL): nanothymoquinone low dose (0.125 µgkg-1 body weight, i.p.) + CCl4 + diet/water, Group IV (NTQM): nanothymoquinone medium dose (1.25 µg kg-1 body weight, i.p.) + CCl4 + diet/water, Group V (NTQH): nanothymoquinone high dose (12.5 µg kg-1 body weight, i.p.) + CCl4 + diet/water, Group VI (TQ): thymoquinone (12.5 mg kg-1 body weight, i.p.) + CCl4 + diet/water. TQ and NTQ (NTQL, NTQM, and NTQH) were administered intraperitoneally for 7 days. Hepatotoxicity was induced in the II, III, IV, V and VI groups by an injection of CCl4 (1.2 mlkg-1, 1: 1 with olive oil, s.c.) on the 3rd and 4th days.

**Preparation of NIPAAM polymeric nanoparticles (pNIPAAM):**

NIPAAM and Acrylic acid (AA) co-polymer were synthesized *via* free radical polymerization. Monomers of NIPAAM and AA were dissolved in water, and the polymerization was carried out under nitrogen (N2) atmosphere. About 90 mg of NIPAAM, and 5 µl AA were dissolved in 10 ml of distilled water. The dissolved oxygen was removed by passing N2 gas for about 45 min. Then, about 25 µl of ferrous ammonium sulphate (FAS) and 35 µl ammonium persulphate (APS) solution were added to initiate the polymerization reaction. The polymerization was carried out at 32°C under N2 atmosphere for about 18 h. On completion of polymerization, the polymeric aqueous solution was dialyzed for about 48 h using a Spectropore membrane dialysis bag (celluSep, 12 KD cut off) with a continuous change of distilled water every 4 h. After this, the aqueous solution was lyophilized to obtain a dry powder of pNIPAAM.

**Surface modification of pNIPAAM with PAG to obtain PAG-pNIPAAM:**

The conjugation of pNIPAAM nanoparticles with PAG was carried out by the carboxyl-amine reaction. Briefly, about 50 mg of lyophilized powder of pNIPAAM was dispersed in 10 ml of double distilled water, and about 10 mg PAG was added to it and allowed to pre-adsorb for 2 h at room temperature. After the pre-adsorbtion, about 0.5 mg of EDC–HCL, a coupling agent for the carboxyl-amine conjugation reaction, was added to the solution described above and the reaction was carried out for about 4 h under continuous stirring at 25°C. The resulting PAG conjugated nanoparticles aqueous solution was dialyzed for 12 h using a Spectropore membrane dialysis bag (celluSep, 12 KD cut off) with continuous change of distilled water every 4 h. After this, the aqueous solution was lyophilized to obtain a dry powder of PAG-pNIPAAM.

**Drug loading**

Thymoquinone (TQ) drug was physically entrapped inside the hydrophobic core of the PAG-pNIPAAM after the complete polymerization reaction. Briefly, about 50 mg of PAG-pNIPAAM lyophilized powder was dispersed in 10 ml of double distilled water. Then, TQ solution in chloroform (5 mg/ml) was gradually added to the dispersed mixture under continuous stirring at room temperature till no settling of drug was observed. The drug-loaded PAG-pNIPAAM was lyophilized to obtain dried powder product for further use.

**CHARACTERIZATION**

Dynamic light scattering (DLS) analysis shows that TQ loaded PAG-pNIPAAM nanoparticles have an average size of about 108.5 nm.

Transmission electron microscopy (TEM) shows that TQ loaded PAG-pNIPAAM nanoparticles have an average size of around 90.0 nm with spherical morphology.

**BIOLOGICAL ACTIVITY**

**Effect of treatment of TQ and NTQ on lipid peroxidation**

Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid per-oxidation (*i.e.* as degradation products of fats), and accordingly, act as an indirect indicator of lipid peroxidation of polyunsaturated fatty acid (PUFA) of membranes of tissues. TBARS was measured to demonstrate the oxidative damage on lipid peroxidation in CCl4-induced liver injury of wistar rats (See, Table 1).

**Table 1: Effect of TQ and NTQ on lipid peroxidation**

|  |  |
| --- | --- |
| **Treatment Groups** | **Lipid peroxidation (µmole TBARS formed/hr/g tissue])** |
| **Group I** – saline (Normal, **N**) | 1.36 ± 0.0203 |
| **Group II** – CCl4 alone (1.20 ml/kg, 1:1 with oil, Disease, **D**)  | 2.55 ± 0.018## |
| **Group III** – CCl4 + **NTQL** (0.125 µg/kg, i.p.) | 2.22 ± 0.045NS |
| **Group IV** – CCl4 + **NTQM** (1. 25 µg/kg, i.p.) | 1.78 ± 0.013\*\* |
| **Group V** – CCl4 + **NTQH** (12.5 µg/kg, i.p.) | 1.45 ± 0.011\*\* |
| **Group VI** – CCl4 + **TQ** (12.5 mg/kg, i.p.) | 1.51 ± 0.014\*\* |

Results represent mean ± SEM of six animals per group. Results obtained are significantly different from control group (\*\*P < 0.01). Results obtained are significantly different from TQ and NTQ treated group (#P < 0.05, ##P < 0.01 and NS P > 0.05). TQ = thymoquinone; NTQL = nanothymoquinone, low dose; NTQM = nanothymoquinone, medium dose; NTQH = nanothymoquinone, high dose

**Effect of TQ and NTQ on the activity of antioxidant enzymes like GPx and Catalase**

Glutathione peroxidase (GPX) and Catalase are one of the most important enzymes in the cell antioxidant defense system. These enzymes collectively act against the free radicals to resist their damaging effects on vital biomolecules and ultimately body tissues. The effect of treatment of TQ and NTQ on GPx and Catalase activity is shown herein-below in Table 2.

**Table 2: Effect of TQ and NTQ on GPx and Catalase activity**

|  |  |  |
| --- | --- | --- |
| **Treatment Groups** | **GPx (nmol NADPH oxidized/min/ mg protein)** | **Catalase (µmol H2O2 consumed/min/mg protein** |
| **Group I** – saline (Normal, **N**) | 462.08 ± 3.92 | 35.32 ± 1.86 |
| **Group II** – CCl4 alone (1.20 ml/kg, 1:1 with oil, Disease, **D**)  | 109.47 ± 3.2## | 8.077 ± 0.43## |
| **Group III** – CCl4 + **NTQL** (0.125 µg/kg, i.p.) | 156.83 ± 3.48\*\* | 9.850 ± 0.00NS |
| **Group IV** – CCl4 + **NTQM** (1. 25 µg/kg, i.p.) | 212.67 ± 2.3\*\* | 14.07 ± 0.54\*\* |
| **Group V** – CCl4 + **NTQH** (12.5 µg/kg, i.p.) | 390.08 ± 7.29\*\* | 28.061 ± 1.47\*\* |
| **Group VI** – CCl4 + **TQ** (12.5 mg/kg, i.p.) | 348.26 ± 3.4\*\* | 22.87 ± 0.79\*\* |

Results represent mean ± SEM of six animals per group. Results obtained are significantly different from control group (\*\*P < 0.01). Results obtained are significantly different from TQ and NTQ treated group (#P < 0.05, ##P < 0.01 and NS P > 0.05). TQ = thymoquinone; NTQL = nanothymoquinone, low dose; NTQM = nanothymoquinone, medium dose; NTQH = nanothymoquinone, high dose