Genotoxicity and Cytotoxicity of Nintedanib Exposure in Germ Cells of Mice: Quercetin from Onion Extracts Intervention

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Abstract

Nintedanib (NTD) is an orally bioavailable, indolinone-derived, receptor tyrosine kinase (RTK) inhibitor with potential antiangiogenic and antineoplastic activities. However, cases of its toxicity have been reported (Motoyasu., et al. 2019). Hence, this study focused on investigating the genotoxic and cytotoxic effects of Nintedanib and possible protective roles of onion (Allium cepa) extract (OE) and quercetin (QUC) in-vivo. Experimental mice were randomly divided into eight groups (n = 5). Group I served as control, group II and III received 10 mg/kg/bwt QUC and OE; groups IV, V and VI were treated with 5, 10 and 20 mg/kg/bwt NTD respectively while 20 mg/kg/bwt NTD were co-treated with 10 mg/kg/bwt of QUC and OE in group VII and VIII respectively via intraperitoneal route for fourteen days. Effect of Nintedanib and potential ameliorative effect of quercetin and onion extract were evaluated in testicular tissue of mice by measuring enzymatic and non-enzymatic oxidative stress biomarkers. Malondialdehyde (MDA) and reactive oxygen species (ROS) levels were measured using spectrophotometry methods. Sperm count, motility and sperm head morphology; viability cells, sperm comet and halo assay and histology of testes were also evaluated. Nintedanib treatments caused reduction epididymal sperm count, motility; viability of spermatozoa cells with increased sperm morphological abnormalities in dose-dependent pattern, confirming the cytotoxicity of NTD following its exposure in mice, however, significant recovery was seen in the groups co-treated with quercetin and onion extract. Induction of oxidative stress was confirmed by raised reactive oxygen species and MDA levels when compared with the control; quercetin and onion extract treatments significantly (p < 0.05) improved the anti-oxidative biomarkers as compared to only NTD-treated mice. The result of comet assay showed that QUC and OE treatments significantly restored the sperm DNA damage induced by Nintedanib. Further, these flavonoid treatments showed protection against NTD-induced testicular toxicity as evident from testes histology. This study has shown that quercetin and onion extract could proffer a measure of protection against cytotoxicity and genotoxicity caused by nintedanib-treatment which lead to testicular oxidative damage by increasing the antioxidant defense mechanism in mice.

Keywords: Nintedanib; Quercetin; Onion Extract; Genotoxicity; Cytotoxicity; Germ Cell

Introduction

Nintedanib is a member of the class of oxindoles that is a kinase inhibitor used (in the form of its ethylsulfonate salt) for the treatment of idiopathic pulmonary fibrosis and cancer. It has a role as an antineoplastic agent, a tyrosine kinase inhibitor, a vascular endothelial growth factor receptor antagonist (VEGFR), a fibroblast growth factor receptor antagonist (FGFR) and an angiogenesis inhibitor with potential antiangiogenic activities. It is an aromatic ester, a methyl ester, a member of oxindoles, an enamine, an aromatic amine, an aromatic amide and a N-alkylpiperazine. Nintedanib targets multiple receptor tyrosine kinases and non-receptor tyrosine kinases.

Quercetin is a flavonoid group of polyphenols. It is found in many fruits, vegetables, leaves, seeds and grains; red onions and kale are common foods containing appreciable content of quercetin. It is used as an ingredient in dietary supplements, beverages and foods. It is
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a naturally occurring polar auxin transport inhibitor [1]. The bioavailability of quercetin in humans is low and highly variable (0 - 50%) and it is rapidly cleared with an elimination half-life of 1 - 2 hours after ingesting quercetin foods or supplements [2]. Quercetin has been reported to inhibit the oxidation of other molecules and hence is classified as an antioxidant [3,4]. It contains a polyphenolic chemical substructure that stops oxidation by acting as a scavenger of free radicals that are responsible for oxidative chain reactions [5]. Quercetin also activates or inhibits the activities of a number of proteins [6]. For example, quercetin is a non-specific protein kinase enzyme inhibitor [3,4]. Quercetin has also been reported to have estrogenic (female sex hormone-like) activities by activating estrogen receptors. In human breast cancer cell lines, quercetin has also been found to act as an agonist of the G protein-coupled estrogen receptor (GPER) [7,8].

Onion extract has a beneficial effect on disease treatment worldwide and has been used since ancient times as a medicinal and food source [9]. *Allium cepa* is commonly used in our daily diet and has been source of much interest because of its antithrombotic, hypolipidaemic, hypotensive, diaphoretic, antibiotic, anti-diabetic, antiatherogenic and anticancer medicinal properties [10]. The biological action of onion is ascribed to organo-sulfur and phenolic compounds it contains [9]. Research has shown that onion contains exogenous and endogenous antioxidants such as selenium, glutathione, vitamins A, B and C and flavonoids such as quercetin [11]. These antioxidants protect DNA and other important molecules from oxidation and damage, which would otherwise induce apoptosis, and could improve sperm health parameters, increasing the rate of fertility in men [12]. Therefore, this study was designed to investigate genotoxicity and cytotoxicity effect of Nintedanib on male reproductive organ and potential protective effect of onion extract and quercetin.

**Methodology**

**Extraction and Soxhlet polar fractionation of onion extracts**

Fresh onions (*Allium cepa* L., Nigeria) were purchased from a local market. The onions were washed with sterile distilled water and allowed to air dry for 24 hour. Method described by Guo-Qing Sh., *et al.* (2016) was used for these processes. Briefly, two hundred grams of onion skin powder was extracted with 6L of 50% aqueous ethanol at 72°C for 2 hours in a water bath. The resulting extract was then cooled to room temperature (25 ± 2°C) and filtered through filter paper. This procedure was repeated two times and the combined extracts were concentrated under vacuum on a rotary evaporator near to cream, which was absorbed onto diatomaceous earth and dried in the air. For soxhlet polar fractionation, the dried diatomaceous earth samples were sequentially extracted with ethyl acetate using a soxhlet extractor for 12 hours. The ethyl acetate extracts were collected for further processing. The ethyl acetate phase was extracted three times with 2% (w/v) NaHCO₃ solution and the pH of the combined aqueous extracts was adjusted with concentrated hydrochloric acid to pH 2. The acidified aqueous was then extracted with ethyl acetate before being evaporated to dryness under vacuum to give the NaHCO₃ components of the ethyl acetate phase which was extracted with 5% (w/v) Na₂CO₃.

**Animals, grouping and treatments**

All the animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC) of College of Medicine, Ekiti state University, Ado-Ekiti, Nigeria. Experiments were performed on male Swiss mice (25g) procured from the central animal facility of the institute. The animals were kept at room temperature (25 ± 2°C), with 50 ± 10% humidity and a cycle of 12h light and 12h dark. Standard laboratory animal feed and water were provided *ad libitum*. Animals were acclimatized to the experimental conditions for a period of one week before the start of dosing.

**Experimental design**

Forty Swiss Albino mice weighing 25 kg were used for this study. The animals were randomly divided into eight groups I-VIII (n = 5). 10 mg/kg Quercetin extract from onion was administered to the Nintedanib (NTD)-treated animal, amount that is the equivalent in ratio to a 25 kg mouse via intraperitoneal route for fourteen days. Experimental mice were weighed before and at the end of the administration

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of the drug. After the treatment, mice were autopsied and testis were excised and stored properly for the analyzes. Below is the table 1 showing the groups and treatments.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Treatment (Dosage/Kg body /weight/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Vehicle-treated control</td>
</tr>
<tr>
<td>Group II</td>
<td>10 mg/kg/day, QUC</td>
</tr>
<tr>
<td>Group III</td>
<td>10 mg/kg/day, Onion extract</td>
</tr>
<tr>
<td>Group IV</td>
<td>5 mg/kg/day, NTD</td>
</tr>
<tr>
<td>Group V</td>
<td>10 mg/kg/day, NTD</td>
</tr>
<tr>
<td>Group VI</td>
<td>20 mg/kg/day, NTD</td>
</tr>
<tr>
<td>Group VII</td>
<td>20 mg/kg/day, NTD + 10 mg/kg/day Onion extract</td>
</tr>
<tr>
<td>Group VIII</td>
<td>20 mg/kg/day, NTD + 10 mg/kg/day QUC</td>
</tr>
</tbody>
</table>

*Table 1: Administration of quercetin, onion extract and NTD.*

Testicular cells preparation

Testicular cells were prepared following a protocol adapted from Malkov, *et al.* [13]. Briefly, testes were removed and decapsulated by making a small incision in the testis. The contents of the testes were collected through the incision into a 15 ml tube containing 5 ml ice-cold 1X PBS buffer (pH-7.4) and the contents were incubated for 40 minutes at 37°C with vigorous shaking. Then, the tubes were placed on ice and incubated to allow the seminiferous tubules to settle. The supernatants were discarded and the seminiferous tubules were washed twice in 10 ml of PBS twice.

Testicular cell viability assay

Cell numbers were determined using a Neubauer hemocytometer and viable cells were analyzed by determining their ability to exclude the dye. The cytoplasm of a viable cell is clear, whereas a non-viable cell has a blue cytoplasm. We calculated cell viability (%) as follows: Cell viability (%) = [total number of viable cells (trypan blue-negative) cells]/[total number of cells including trypan blue-positive and negative cells]* 100.

Measurement of reactive oxygen species (ROS) level

The ROS assay was performed by the method of Hayashi, *et al.* [14]. In brief, 50 µl of tissue homogenate and 1400 µl sodium acetate buffer were transferred to a cuvette. After then, 1000µl of reagent mixture (N,N-diethyl para phenylenediamine 6 mg/ml with 4.37 µM of ferrous sulfate dissolved in 0.1M sodium acetate buffer pH- 4.8) was added at 37°C for 5 minutes. The absorbance was measured at 505 nm using spectrophotometer (Molecular Devices.) ROS levels from the tissue were calculated from a calibration of H₂O₂ and expressed as U/mg of protein (1 unit = 1.0 mg H₂O₂/L).

Measurement of malondialdehyde level

For this purpose, one gram of testicular tissue was homogenized in 0.05M phosphate buffer at pH = 7.4 and the concentration of 10% (w/v). Then obtained solutions were centrifuged in 1000g and supernatants were used for the evaluation of levels of lipid peroxidation products. Spectrophotometric measurement method based on the color produced by reaction of TBA with MDA was used. 300 ml of trichloric acid 10% was added to 150 µl supernatant of centrifuged sample and then centrifuged for 10 minutes at 4°C and 1000g. 300 µl of supernatant were transferred to a test tube and was incubated with 300 µl of thiobarbituric acid 0.67% at 100°C for 25 minutes. 5 minutes after cooling the solution, the pink color due to the reaction of TBA-MDA appeared and was measured using a spectrophotometer at a
wavelength of 535 nm. Concentration of MDA was calculated using the coefficient of TBA-MDA complex absorption and was expressed as nmol/g wet tissue [15].

**Glutathione (GSH) level**

Glutathione (GSH) activity was estimated by centrifuging an aliquot of 10% homogenates of the tissues in 100 mM Tris-HCL buffer (pH-7.4) containing 0.16M KCL at 1000 × g for 5 minutes. The supernatant was used to measure the rate of reduction of 5′5′-dithiobis-(2 nitrobenzoate) to 2-nitro-5 thiobenzoate. The absorbance was taken at 412 nm. Glutathione content was expressed in µM/mg protein [16].

**Superoxide dismutase (SOD) level**

Superoxide dismutase (SOD) was measured using modified method of Kakkar, et al [17]. Assay mixture containing sodium pyrophosphate buffer (pH-8.3, 0.052M), nitroblue tetrazolium (300 µM), NADH (780 µM) and appropriately diluted enzyme in total volume of 3 ml was incubated at 37°C for 90 seconds. The reaction was stopped by the addition of glacial acetic acid. The reaction mixture was missed vigorously by adding n-butanol and was allowed to stand for 10 minutes before the collection of butanol layer. The intensity of chromogen in butanol was measured at 560 nm. The SOD activity was calculated in units/ml/min.

**Catalase (CAT) level**

Catalase was measured by the method described by Aebi [18]. Assay mixture consisting of 0.01M phosphate buffer (pH-7.0), 0.2M hydrogen peroxide (H₂O₂) and tissue homogenate was incubated at 37°C for 1 minute. The reaction was stopped by addition of potassium dichromate (5% w/v) and acetic acid. The remaining hydrogen peroxide was determined by measuring chromium acetate after heating the assay mixtures in a boiling water bath for 15 minutes. The absorbance was read at 570 nm against control (without H₂O₂). The enzymatic activity was measured in µmol/min/mg protein.

**Sperm count and sperm head morphology**

After the animal sacrifice, epididymis was removed and placed in a petri-plate containing 2 ml of HBSS medium at room temperature. The epididymis was cut into small portions to allow the sperms to swim out. The solution containing the sperms was centrifuged at 1000 rpm for 3 minutes. After centrifugation, 1 ml of supernatant was taken and used for sperm counting, sperm head morphology and sperm comet assay. The epididymal sperm count was determined by hemocytometer. The sperm count was expressed as number of sperms per millilitre. For sperm head morphology 0.5 ml of above solution containing the sperms and 0.5 ml of 2% eosin solution were mixed and kept for one hour to stain the sperm. Smears were prepared using 2 - 3 drops of the above solution, air dried and fixed with absolute methanol for 3 minutes. Two hundred sperms per animal were examined to determine the morphological abnormalities under oil immersion [19]. Sperm head morphology was scored under the category of normal, quasinormal and grossly abnormal as described by Burruel, et al [20]. Sperms missing rostral part of the acrosome and/or the posterolateral region of the acrosome are called quasi-normal heads. Grossly abnormal heads included collapsed and triangular heads with highly deformed acrosomal caps and nuclei. Data were shown in terms of normal to abnormal ratio of sperms.

**Sperm motility**

In order to observe mobility, 10 microliters of semen was placed on a glass slide and covered with a lamella. Using a light microscope with a magnification of 400×, the number of sperm with rapid progressive forward movement (RPFM), slowly progressive forward movement (SPFM), Non-progressive motility (NPM) and motionless (ML) sperm cells were counted in several microscopic field of vision and percentage of motile and Immobile sperm cells was obtained [21].

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Testis histology

Preparation and quantification of histological slides were done as standardized previously in our laboratory [22,23]. Both the testes were fixed in 10% formalin, dehydrated in ethanol and embedded in paraffin. Tissue sections (5m) were mounted on glass slide coated with albumin and dried at 30°C for 24h. The sections were then deparafinized with xylene, rehydrated with alcohol and water. The rehydrated sections were stained with haematoxylin and eosin (H&E), mounted with DPX and examined under microscope. Histological quantification was performed by counting the normal number of seminiferous tubules in each slide. A graph was plotted between the normal number of seminiferous tubule at Y-axis vs. dose of Nintedanib at X-axis. Relative area was calculated by area of treatment group/area of control group.

Halo assay

The halo assay was performed essentially as described with some modifications [24]. Testis was homogenized gently in PBS and 5 μl of the homogenate was suspended in 50 μl of 0.5% low melting point agarose and layered over the surface of a frosted slide (pre-coated with 1% normal melting point agarose) to form a microgel and allowed to set at 4°C for 5 minutes. The slides were immersed in freshly prepared lysis solution (2.5M NaCl, 2 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100) for 2h at 4°C. Following lysis, the slides were incubated with alkaline medium (0.3M NaOH) for 20 minutes and stained using EtBr. Samples were run in duplicate and 50 cells were randomly examined per slide for a total of 100 cells per sample under the microscope. The damaged cells were categorized as mild, moderate and extensive as described [25].

Single-cell gel electrophoresis (Comet assay)

The alkaline comet assay was performed as described by Singh., et al. [26] to assess DNA strand breaks. Briefly, the testicular cells were mixed with 85 μL of 0.5% low-melting agarose and placed on a microscope slide that was pre-coated with 0.6% normal melting agarose. Another 85 μL of low-melting agarose was added to form the top layer. The cells were lysed at 4°C in an ice-cold, freshly prepared solution of 2.5M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% dimethyl sulphoxide at pH 10. The slides were placed on a horizontal gel electrophoresis unit. DNA was allowed to unwind for 20 minutes in an electrophoretic alkaline buffer (1 mM Na₂EDTA, 300 mM NaOH, pH 13) and electrophoresed in the same buffer for 25 minutes at 0.78 V/cm and 330 mA. Once electrophoresis was complete, the slides were gently washed in a neutralization buffer (0.4 M Tris-HCl, pH 7.5). Subsequently, the slides were dried and fixed with absolute ethanol for at least 60s. Prior to examination, the slides were stained with 100 μL ethidium bromide (10 μL/mL, Sigma-Aldrich). For visualization of DNA damage, observations were made using the 40° objective of a fluorescent microscope. The Olive tail moment (the comet tail length and the DNA content of the tail), defined as the fraction of total DNA in the tail, was used as an index of damage [23].

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 for Windows statistical package (SPSS Inc., Chicago, IL, USA). All experiments were performed in triplicate and the inter-group difference among three experiments was tested by analysis of variance (ANOVA). Data are expressed as means ± SD or SEM of three different experiments, and p < 0.05 was considered statistically significant.

Results

The effect of quercetin and onion extract on cell variability

In the line graph below, the co-treatment of Nintedanib with Quercetin and Onion (groups VII and VIII) extract a protective effect on the cell variability of Nintedanib-treated cell (group IV, V and VI), as there was signs of recovery in the cell variability of the groups VII and VIII.

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Whereas, there was a significant decrease in the cell variability of group VII and VIII, compared to groups I, II and III. Also, a significant decrease was evident in Group IV, V and VI compared to that seen in Group I, II and III (Figure 1).

Protective effect of onion extract and quercetin on ROS level

Nintedanib-treatment led to a significant increase in the Reactive Oxygen Species (ROS) level in the testicular tissues of the mice in Groups IV, V and VI compared to the control, QUC and OE (Groups I, II and III). Co-treatment of nintedanib with Quercetin and Onion extract (Groups VII and VIII) led to a significant decrease in the ROS level in the testicular tissues of the mice in Groups VII and VIII compared to Groups IV, V and VI, whereas a sign of recovery was shown as there was no significant difference in the testicular cells in Groups VII and VIII compared to Groups I, II and III (Figure 2).
The level of Malondialdehyde (MDA) following nintedanib treatment in the mice in Groups IV, V and VI were significantly increased compared to Groups I, II and III. Co-treatment of nintedanib with onion extract and quercetin exerted a significant increase in the testicular tissues of the mice in Groups VII and VIII compared to Groups I, II and III, whereas no significant difference was shown in the level of Malondialdehyde in the testicular tissues of the mice in Groups VII and VIII when compared to Groups I, II and III (Figure 3).

**Figure 3:** The effect of Onion extract and Quercetin on the Malondialdehyde (MDA) level of Nintedanib induced cells. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.

The protective effect of onion extract and quercetin on reduced glutathione (GSH) level

A significant decrease (p < 0.05) was recorded in the Glutathione level of Nintedanib-treated testicular tissues in the mice in Groups IV, V and VI compared to the control, QUC and OE (Groups I, II and III). Co-treatment of nintedanib with Onion extract and Quercetin exerted a significant increase in the Glutathione level of Nintedanib-treated testicular tissue (Groups VII and VIII) compared to Groups IV, V and VI (Figure 4).

**Figure 4:** The effect of Onion extract and Quercetin on the Glutathione (GSH) level of Nintedanib induced cells. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.
Effect of onion extract and quercetin on the SOD

Nintedanib treatment led to a significant increase in the superoxide dismutase (SOD) level of the testicular tissue in Groups IV, V and VI compared to Groups I, II and III. Co-treatment of nintedanib with Onion extract and Quercetin (Groups VII and VIII) shows signs of recovery as there was no significant difference recorded in Groups VII and VIII compared to Groups IV, V and VI, whereas, there was a significant increase compared to Groups I, II and III (Figure 5).

![Figure 5: The effect of Onion extract and Quercetin on the Superoxide dismutase (SOD) level of Nintedanib induced cells. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.](image)

Protective effect of onion extract and quercetin on the catalase activity

The levels of catalase (CAT) in nintedanib-treated testicular cells (Group IV, V and VI) was significantly reduced compared to the control, QUC and OE (Groups I, II and III). Co-treatment of Nintedanib with Onion extract and Quercetin, shows signs of recovery as there was no significant difference in the catalase level of the testicular tissues in the mice (Group VII and VIII) compared to the nintedanib-treated cells (Group IV, V and VI) whereas there was a significant decrease in catalase level of the testicular tissue of the mice in Groups VII and VIII compared to Groups I, II and III (Figure 6).

![Figure 6: The effect of Onion extract and Quercetin on the Catalase (CAT) level of Nintedanib induced cells. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.](image)
The effect of quercetin and onion extract on sperm count

Nintedanib treatment at the doses administered in Group IV, V and VI led to a slight decrease in sperm count with increasing dosage, as compared to Group I, II and III. Co-treatment of Nintedanib with Quercetin and Onion extract (Group VII and VIII), led to a slight increase in the sperm count of the group (VII and VIII) as compared to that in the Nintedanib-treated cells. Quercetin and Onion extract treatment on testicular cell, restored normal sperm count, as compared to the control (Figure 7).

![Figure 7](image1.png)

**Figure 7:** The effect of Quercetin and Onion extract on sperm count of Nintedanib induced cell. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.

The effect of quercetin and onion extract on sperm morphological

The incidences of morphologically abnormal sperm in the testicular tissues of all the Nintedanib-treated cells (Group IV, V and VI) were significantly increased compared with that in Group I, II and III. Co-treatment of Nintedanib with Quercetin and Onion extract (Group VII and VIII) showed a significantly decreased incidence compared with the Nintedanib-treated cells (Group IV, V and VI) whereas an increase was seen compared to Group I, II and III. Therefore, Quercetin and Onion extract treatment significantly prevented the damage induced by Nintedanib and restored normal sperm morphology (Figure 8).

![Figure 8](image2.png)

**Figure 8:** The effect of Quercetin and Onion extract on sperm morphological abnormalities in Nintedanib induced cells. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.
Histology observation of testis

Nintedanib-treatment (20 mg/kg) on the testicular tissues caused a significant loss of leydig cells, due to the degradation of germ cells in the seminiferous tubule of the nintedanib-treated mice. Co-treatment of nintedanib with onion extract shows signs of recovery as there was a significant restoration of the seminiferous tubule and in the rate of leydig cell loss in the testicular tissues of the mice treated with ND and Onion extract (20 and 10 mg/kg respectively).

Figure 9: Photomicrographs of mice testis stained with haematoxylin and eosin (H&E). (A) Control (normal saline), (B) ND treatment (20 mg/kg) and (C) ND + (Onion extract (20 and 10 mg/kg respectively.”#” indicates the degeneration (b- ND-treatment alone) and restoration of seminiferous tubule (co-treatment with Onion Extract).

Ameliorative potential of onion extract and QUC on % damaged cells by halo assay

The ameliorative potential of onion extract and quercetin on percentage damaged cells following nintedanib treatment show a significant increase in group VII and group VIII, compared to group I, group II and group III which is similar to that in group IV, group V and group VI, compared to group I, group II and group III, in both mild and extensive indices. Also no significant difference is recorded in group VII and group VIII, compared to group IV, group V and group VI, in both mild and extensive indices (Figure 10).

Figure 10: Ameliorative potential of Onion extract and QUC on % damaged cells following ND- treatment determined by halo assay in testis. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. Nintedanib -20 mg/kg.

Effect of quercetin and onion extract on induced sperm DNA damage by comet assay

The effect of quercetin and onion extract on nintedanib induced sperm DNA is shown in table 2. The tail length in group VII (20 mg/kg/day ND + 10 mg/kg/day OE) and group VIII (20 mg/kg/day ND + 10 mg/kg/day QUC) shows signs of recovery due to the co-treatment of nintedanib with onion extract and quercetin, as there was no significant difference compared to the nintedanib treated groups (II, III and IV). Those in group VI (QUC 10 mg/kg) and group V (OE 10 µl/ml), were significantly decreased. Also, the percentage DNA in Tail of group V and group VI shows a significant decrease, compared to the groups co-treated with ND, OE and QUC (groups VII and VIII), which shows signs of recovery as there was no significant difference compared to groups II, III and VI. Furthermore, the olive tail movement observed in group VII and group VIII were significantly increased compared to groups II, III and IV and also groups V and VI. Finally, the tail movement seen in the group VII and VIII shows signs of recovery as there was a slight increase in the tail movement in the group compared to that in groups II, III and IV whereas group V and group VI were significantly decreased. Therefore, Quercetin and Onion extract treatment significantly increase the DNA damage on Nintedanib induced sperm DNA as evident by the comet parameters.

<table>
<thead>
<tr>
<th>Dose groups</th>
<th>Tail length µm</th>
<th>%DNA in Tail</th>
<th>Olive tail movement</th>
<th>Tail movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9 ± 0.011</td>
<td>11.36 ± 1.06</td>
<td>5 ± 0.04</td>
<td>11 ± 0.12</td>
</tr>
<tr>
<td>5 mg/kg/day, ND</td>
<td>15 ± 0.14</td>
<td>20.3 ± 0.02</td>
<td>3 ± 0.11</td>
<td>22 ± 0.08</td>
</tr>
<tr>
<td>10 mg/kg/day, ND</td>
<td>36 ± 0.34</td>
<td>29.78 ± 0.02</td>
<td>3 ± 0.07</td>
<td>39 ± 0.16</td>
</tr>
<tr>
<td>20 mg/kg/day, ND</td>
<td>42 ± 0.21</td>
<td>37.5 ± 0.04</td>
<td>6 ± 0.15</td>
<td>52 ± 0.45</td>
</tr>
<tr>
<td>QUC (10 mg/kg)</td>
<td>7 ± 0.07</td>
<td>11.57 ± 0.16</td>
<td>9 ± 0.05</td>
<td>11 ± 0.22</td>
</tr>
<tr>
<td>Onion Extract (10 µl/ml)</td>
<td>9 ± 0.05</td>
<td>12.7 ± 0.01</td>
<td>10 ± 0.12</td>
<td>10 ± 0.01</td>
</tr>
<tr>
<td>20 mg/kg/day, ND + 10 mg/kg/day Onion extract</td>
<td>37 ± 0.22</td>
<td>20.79 ± 0.16</td>
<td>13 ± 0.13</td>
<td>27 ± 0.18</td>
</tr>
<tr>
<td>20 mg/kg/day, ND + 10 mg/kg/day QUC</td>
<td>32 ± 0.15</td>
<td>23.53 ± 0.17</td>
<td>8 ± 0.14</td>
<td>36 ± 0.04</td>
</tr>
</tbody>
</table>

**Table 2:** Effect of QUC and Onion extract on ND induced sperm DNA damage as revealed by comet assay.

**Figure 11:** Photomicrographs showing the DNA migration pattern of sperm nuclei in single cell gel electrophoresis (comet) assay stained with Hematoxylin (A) Sperm nuclei from control (normal saline), (B) sperm nuclei from ND treatment (20 mg/kg of ND) and (C) ND + Onion Extract.

Discussion

The present study which aimed at evaluating the genotoxic and cytotoxic effects of onion extract and quercetin on nintedanib-induced germ cells, explored their effects on different biochemical and morphological parameters which includes sperm count, sperm morphology, sperm cell variability, spermatogenesis, apoptosis and oxidative stress.

Quercetin is a plant flavonol from the flavonoid group of polyphenols found in food, commonly in red onion. Quercetin has been reported to inhibit the oxidation of other molecules and hence is classified as an antioxidant and it also activates or inhibits the activities of a number of proteins. Studies have shown that flavonoids such as quercetin 4-O-glucoside and quercetin 3,4-O-diglucoside are the major compounds found in onion and compounds derived from kaempferol andisorhamnetin were identified as minor flavonoids [27]. Another study by Arabbi., et al. (2004) has also shown that significant amounts of quercetin aglycone are found in concentrations of 48 - 56 mg/100g on white onions and amounts of 38 - 94 mg/100g in red onions. Onion extract contains exogenous and endogenous antioxidants that protect DNA and other important molecules from oxidation and damage. It also contains flavonoids such as quercetin.

Oxidative stress results from excessive biosynthesis or intake of pro-oxidants, impaired bio-synthesis of antioxidants or a combination of both [28]. Balancing pro-oxidant and anti-oxidants is vital for normal testis function and sperm fertilization ability [29]. Thus, excessive generation of reactive oxygen species can cause cellular damage and impair sperm structure and function [28].

In this study, the anti-oxidant enzyme activities of sodium dismutase (SOD), catalase (CAT), Malondialdehyde (MDA), Glutathione (GSH) and Reactive Oxygen Species (ROS) levels were assessed. Nintedanib treatment caused a significant increase in MDA and a significant decrease in CAT activity. This observation could be responsible for the poor sperm quality associated with nintedanib-induced mice since reactive oxygen species (ROS) have been proposed to have a role in gametogenesis [28]. A decrease in the serum level of CAT indicates oxidative stress as CAT is known to convert hydrogen peroxide produced in a cell to oxygen and water thus, completing the detoxification process initiated by SOD [30]. This result is in consonance with the work of Weyers., et al. [31] who observed that there was an increase in peroxide radical generation following ciprofloxacin treatment in mice. Vartan., et al. [32], Khaki., et al. [33] and Abu-Aita., et al. [34] reported similar observations. Onion extract and was found to maintain the level of CAT close to the control when administered together with nintedanib. The anti-oxidant property of onion extract observed in this study agrees with the work of Khaki., et al. [9], Ige and Akhigbe [35].

In this study, low dose of onion extract and quercetin maintained the protein content of the germ cell close to that of the control while treatment with nintedanib with or without high dose of onion extract led to a non-significant decrease in the protein content compared to the control. This result is in disagreement with the work of Fatali., et al. [36] who observed a statistically significant increase in the level of serum total protein concentration of mice following administration of ciprofloxacin.

In this study, we examined to determine whether quercetin and onion extract would have an alleviative effect against male reproductive toxicity of Nintedanib-treated testicular cell. Treatment with onion extract and quercetin was found to potentiate the toxic effect of Nintedanib on sperm count, sperm motility and sperm cell viability. This observation agrees with the work of Abd-Allah., et al. [37], Vartan., et al. [32], Abu-Aita., et al. [34] and Elias and Nelson [38] who reported that ciprofloxacin (an anti-cancer drug) administration produced significant reduction in sperm count, sperm motility and sperm viability. Also, our result clearly indicates that quercetin and onion extract improved the increasing coefficient of abnormal morphological sperm. This is in agreement with work of Hiromi Izawa., et al. [39] who reported that quercetin and onion extract ameliorate the increased indices of abnormal sperm and decreased the number of stained sertoli cells induced by diesel exhaust particles (DEPs). Further, sperm comet assay revealed the genotoxicity of nintedanib as evident from the tail length, tail movement, olive tail movement and percentage DNA in comet tail. Therefore, the percentage of damage experienced on the morphology of sperm following Nintedanib treatment, was alleviated upon administration of Quercetin and Onion extract, a percentage of improvement was recorded, with increasing potential down the group.

Nintedanib-treatment on the testicular tissues caused a significant loss of leydig cells, due to the degradation of germ cells in the seminiferous tubule of the nintedanib-treated mice. Onion extract has a restorative effect on the seminiferous tubule and in the rate of leydig cell loss in the testicular tissues of the mice. This is in line with the work of Izawa., et al. [39] which reported that onion and quercetin has a stabilizing effect on sertoli cells as shown by immunohistochemical staining of the Sertoli cell tubuline (cytoskeletal protein enriched in sertoli cells) by monoclonal anti-tyrosinated tubulin antibody. This reduced the function of sertoli cells for spermatogenesis.

Apoptosis is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alteration leading the cell to suicide [40]. Testicular apoptosis monitors germ cell population to the support capacity of Sertoli cells [28]. Toxicants induce massive germ cell death either by increasing the expression of apoptosis related proteins or by oxidative imbalance [28]. Also, DN Tripathi and GB Jena [23] reported that cyclophosphamide (an anti-cancer drug) exerted its toxic effects in male germ cell mainly due to redox imbalance and oxidative stress, which result in apoptosis. This agrees with the result of this present study which records an increase in apoptosis on the germ cell of nintedanib-treated testicular cells [41-44].

Conclusion

In conclusion, this present study shows that nintedanib is toxic towards induced male germ cell. In addition, the study also indicates the restorative effect of onion extract and quercetin against nintedanib induced male germ cell toxicity in mice. More work with both animal models and in vitro human germ cell lines are needed, to explore possible mechanisms of onion extract and quercetin potential which can facilitate to reduce testicular toxicity in patient been treated with nintedanib.

Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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