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Effect of *Andrographis paniculata* on p53 expression during cisplatin induced toxicity in rats

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Abstract
The study involved the effect of *Andrographis paniculata* extract (APE) on p53 expression in liver of cisplatin treated rats. Experimentation procedure involves 5 groups with 12 Wistar albino rats in each group. Rats of Positive cisplatin control receive single injection of cisplatin (7.5mg/kg, Intraperitoneal route) only. The concurrent and pretreatment control rats receive cisplatin on first day and APE (500mg/kg, Oral gavage) on first day and its treatment continue up to 45 days. But the pretreatment control rats receive the APE for 15 days prior to cisplatin treatment. The normal control and extract control rats receive only normal saline and APE respectively during experimental period. The p53 quantification and immunohistochemical analysis were performed on liver samples which were collected from 7th, 14th, 28th and 45th day of experiment.
The results revealed a significant increase in p53 gene expression in the cisplatin control rats (p<0.05), whereas all APE treated rats showed a significant decrease in p53 expression (p<0.05) as indicated by relative quantification. Immunohistochemical analysis of liver sections demonstrated a higher number of p53-positive cells in the cisplatin control. In contrast, the APE treated control rats showed a marked reduction in both fold expression and the number of cells stained for p53. So, APE may exert anti-apoptotic and antioxidant effects potentially mitigating the cellular damage caused by cisplatin.

Keywords: Cisplatin, antioxidant, hepatotoxicity, *Andrographis paniculata*, p53

Introduction
Cisplatin is most used anti cancerous drug used for the treatment of commonly occurring tumors in humans. It is DNA damaging agent and closely interact with DNA molecules and form intra and inter stand adducts [1]. Several toxic effects induced by cisplatin on several organs including liver along with decrease in oxidative enzymes causing oxidative stress [2]. The treatment of cisplatin at different experimental dosages causes hepato and nephrotoxicity as per the previous reports [3, 4, 5].
Now days, for reduction and amelioration of toxic side effects induced by anti-cancerous drugs, uses several phytochemical and herbal plants. Among that, *Andrographis paniculata* is widely used around the world and it is named differently as Creat, Kiriyat, Kalmegh and Nelaberu in different languages[6]. It has been reported by several workers as hepatoprotective, nephroprotective, antioxidant and anti-inflammatory properties [7, 8, 9]. The composition of phytochemicals in *Andrographis paniculata* include diterpenes, lactones, flavoids and andrographolide [10].
The apoptosis is most important mode cell of death involving both physiological and pathological processes. The number of causative factors such as chemotherapeutic drugs, cytotoxic drugs, radiation, viruses, autoimmune diseases and others can induce apoptosis. Apoptosis due to cisplatin treatment in experimental rats and mice has been reported previously [11, 12, 13]. Cisplatin cytotoxicity involves death of cells by both necrotic and apoptotic pathways. Hence to elucidate p53 mediated apoptosis, in the current study expression of p53 was evaluated through immunohistochemistry and real time PCR on liver tissues and its amelioration by *Andrographis paniculata.*

Materials and Methods

The study involving Cisplatin (Kemoplat, Fresenius Kabi India Pvt. Ltd. Pune,India.), *Andrographis paniculata* ethanolic extract (Himalaya herbal pvt Ltd. Bangalore, India) and other chemicals reagents and kit used were of good quality purchased from local source. The experimental study and design involving 60 Wistar albino rats were maintained under standard laboratory conditions and after acclimatization of 15 days, rats were segregated into five groups with 12 rats in each. "The treatment protocol was carried out based on procedure described previously"[14].

Quantification of p53 expression by real time PCR (q-PCR)

The RNA extracted as per the procedure described by M/s Invitrogen (USA) used for preparation complementary DNA (cDNA) synthesis of p53 and GAPDH genes of rat using gene specific primers as per the standard protocol with the addition of 20 units of MMLV reverse transcriptase and incubated at 42 °C for 60 minutes. In order to assess relative quantity of rat p53 gene, Taqman Real Time PCR assay was carried out using Eppendorf realplex 2 real time PCR detection system (Hamburg, Germany) with ready to use 2X master mix (3B quantimix, Blackbio Biotech India Limited, India). The p53 and GAPDH primer sequences used were as follows: p53-F,5'-TGGGGAATGGGTTGGTAGT-3'andp53-R, 5'-GGTGGGGTGGGGTGAAAT-3'; GAPDH-F,5'-ATGACTCTACCCACGGCAAG-3' and GAPDH-R, 5'-TACTCAGCACCAGCATCACC-3'. For quantification of gene transcription, GAPDH was used as reference gene and the comparative ct (cycle threshold) method was used for fold change in the p53 gene between treatment and normal control group calculated with analyzation of data using statistical software (Graph Pad Prism, version 6.0).

Immunohistochemical detection of p53 expression

The 4µ sections of liver were cut and mounted on to 3-aminopropyltriethoxy-silane (APES) coated slides, deparaffinized, rehydrated and sections were heated under Tris-EDTA buffer (pH 9.0) for unmasking of epitopes from all the paraffin blocks of treatment and control rats for immunohistochemical expression of p53 using monoclonal antibody. Monoclonal mouse anti p53 antibodies (Ready to use) were added to cover sections and were incubated at 37 °C in humidified chamber for 1 hour 30 minutes. Primary antibody used for immunohistochemical evaluation included an Monoclonal mouse anti p53 antibody (Clone-BP-53-12, Pathinsitu, USA). Secondary antibody kit used was the Polyexcel HRP/DAB detection system kit and Poly-target binder to p53 used and incubated as per the protocol described by Pathinsitu (USA). Later sections were washed, diaminobenzidine (DAB) working solution was poured to cover the sections and incubated at room temperature for till desired color developed. Nuclear staining performed using Harris hematoxylin and later sections were processed with dehydration and clearing procedure and mounted with DPX.

Results

Real time PCR for p53 expression in liver

Expression of p53 in liver tissue of animals belonging to different groups was estimated by real time PCR using Taqman primers and probes. Comparative Ct method was used for relative quantification of *p53* gene expression. The housekeeping gene GAPDH was used as internal control and the values were expressed as fold increase/decrease as compared to normal control animals (Table 1)

The table showed increase p53 expression ($p<0.05$) in cisplatin positive control rats compared to APE control, APE concurrent and APE pretreated control rats on all days of observation. The APE concurrent and APE pretreated control rats also showed increase p53 expression ($p<0.05$) compared to APE control rats on 7th, 14th, 28th, and 45th day, but still the values were lesser ($p<0.05$) compared to cisplatin positive controls rats. The APE pretreatment control rats showed more decrease in p53 expression ($p<0.05$) compared to APE concurrent rats all days of observation.

Table 1: The mean fold expression of p53 in liver of rats

Groups	Days of post treatment			
	07th	14th	28th	45th
Positive Cisplatin control	189.39±0.385^{ax}	169.55±0.35^{ay}	138.39±0.18^{az}	43.56±4.90^{aw}
APE Control	0.85±0.03^{bx}	0.25±0.02^{bx}	0.40±0.30^{bx}	0.33±10.58^{bx}
APE pre-treatment	99.58±0.26^{cx}	67.55±0.34^{cy}	53.43±0.28^{cz}	37.53±3.48^{aw}
APE concurrent	173.45±0.17^{dx}	158.62±0.30^{ay}	140.48±0.18^{az}	30.76±0.94^{aw}

Superscripts "a, b, c, d" denotes significant difference between groups at $p<0.05$

Superscripts "x, y, z, w" denote significant difference between days at $p<0.05$

Immunohistochemical demonstration of p53 in liver

In the present study, the expression of p53 gene protein following administration of cisplatin was demonstrated immunohistochemically in different treatment groups. Appearance of brown or dark brown coloured granularity in the nucleus and cytoplasm was considered as positive reaction. The cells which showed nuclear staining were considered as apoptotic cells and those cells which showed only cytoplasmic staining were considered as pre-apoptotic cells. In the animals belonging to normal and APE control, only occasional cells revealed positive staining for p53, comparatively more in APE control, with the granular brown staining restricted to nucleus or cytoplasm. The extent of appearance of apoptotic cells did not vary throughout the experimental period.

Microscopically, p53 positive cells in cisplatin control rats were found distributed throughout the liver parenchyma. On 7th day a large number of p53 positive cells were observed in the liver lobules. The p53 immunoreactivity was cytoplasmic and appeared granular and yellowish brown in colour (Plate 1A). Occasional cells which showed nuclear staining were characterized by the presence of condensed, dark brown coloured nuclei which were round, oval, crescent or dot shaped with scanty brown coloured cytoplasm. The p53 positive reaction was more concentrated in the centrilobular and periportal hepatocytes. On 14th and 28th day also, a large number of p53 immune positive cells were observed similar to those observed on 7th day (Plate

1B, 1C). However, the intensity of colouration and granularity of p53 positive cells appeared slightly reduced. On 45th day a small number of hepatocytes showed intense reaction and many of the cells appeared faintly stained with p53 immunostaining. During 45th day many apoptotic cells with nuclear staining were also observed.

In both the APE concurrent and APE pretreatment control rats, the pre-apoptotic and apoptotic cells positive for p53 were found distributed throughout the liver lobule similar to that observed in cisplatin control rats on 7th day of study. The number and intensity of positive reaction of p53 were lesser than that observed in cisplatin positive control rats but was similar in APE concurrent rats. The p53 expression was observed also on 14th, 28th and 45th day of the study however, there was a decrease in the number and the intensity of immune reactivity in both the groups. Comparatively the p53 expression was more in the APE concurrent than APE pretreatment rats (Plate 1D to 1H).

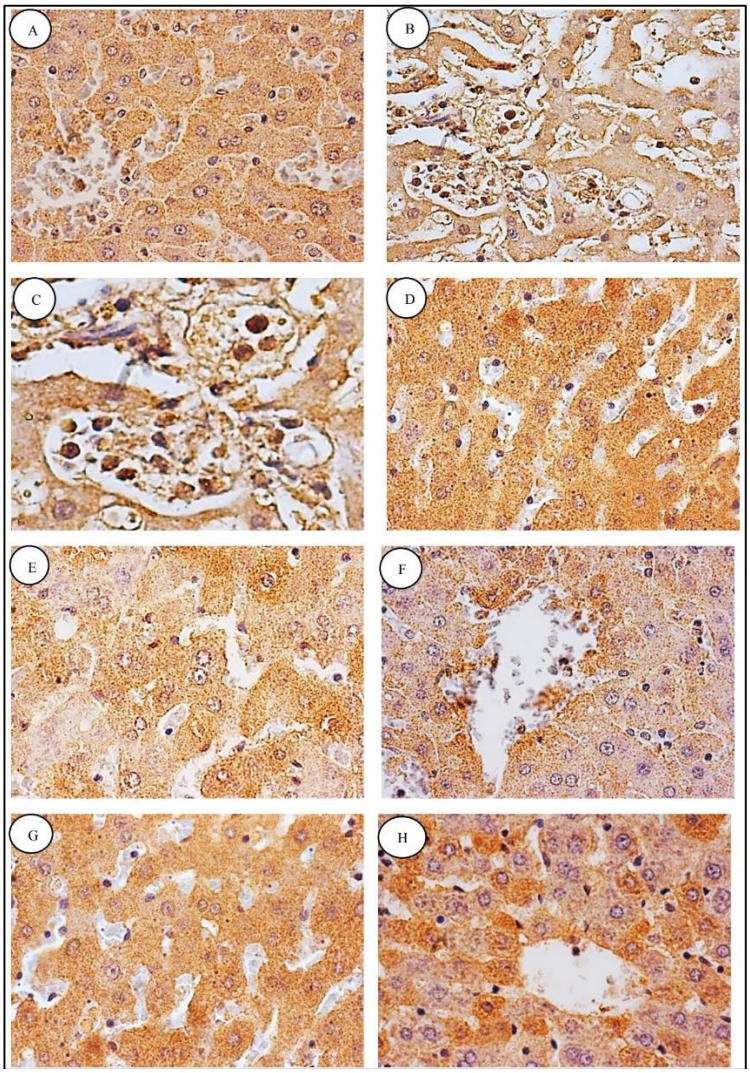

Plate 1: Expression of p53 by immunohistochemical method in liver.

A) Cisplatin controls rat showing expression of p53 in hepatocytes with diffuse cytoplasmic granular staining (IHC ×400).

B) Cisplatin controls rat showing p53 positive apoptotic hepatocytes with cytoplasmic granular staining in the liver at 28th day. Note multifocal areas showing necrotic and apoptotic cell (IHC ×400).

C) Cisplatin controls rat showing a p53 positive apoptotic cells in the liver (IHC ×1000).

D) The APE pretreatment rat showing diffuse p53 positive reaction in hepatocytes at 7th day (IHC×400).

E) The APE pretreatment rat showing reduced expression of p53 in the hepatocytes at 28th day with a few cells negative for p53 staining (IHC ×400).

F) The APE pretreatment rat showing reduced expression of p53 in the hepatocytes on 45th day (IHC ×400.

G) The APE concurrent rat showing p53 positive granularity in the hepatocytes at 28th day (IHC ×400).

H) The APE concurrent rat showing comparatively less number of p53 positive hepatocytes on 45th day (IHC ×400).

Discussion and Conclusion

p53 is a tumour suppressor gene that codes for a protein which regulates the expression of several genes[15]. The physiological function of p53 is prevention of inappropriate cell proliferation and maintenance of genome integrity following genotoxic stress. Following DNA damage by means of ionizing radiations, UV irradiation, cytotoxic drugs or chemotherapeutic agents and infectious virus, p53 activation increases overall p53 protein level resulting in activation of p53 targeted genes.

Cisplatin cytotoxicity involves death of cells by both necrotic and apoptotic pathways. The role of p53, caspase 3, 8, and 9, and mitochondria were investigated in the signaling of cisplatin-induced apoptosis previously[16]. The cisplatin induced alteration of p53 expression in the kidney immunohitochemically using cisplatin induced acute renal

failure [17]. Hence to elucidate whether there is any p53 mediated apoptosis expression of p53 was evaluated through immunohistochemistry and real time PCR on rats liver tissue in the current study.

The relative quantification of p53 expression in liver tissue revealed highest p53 expression (189.39±0.385) folds in cisplatin control rats on 7th day followed by that in APE concurrent (173.45±0.17) and APE pretreatment (99.58±0.265) which were significantly higher compared to APE control rats. The fold expression of p53 in cisplatin control rats was observed to be declined on 45th day (43.56±0.49), however remained higher compared to any other treatment groups. The results of relative fold expression of p53 correlated well with the results of immunohistochemical expression of p53.

The results indicated that the apoptotic death of cells in cisplatin induced hepatotoxicity was p53 mediated and treatment with *Andrographis paniculata* significantly reduced the p53 expression in the APE pre-treatment rats compared to concurrent treatment rats. This could be attributed to the antioxidant phytochemicals present in the APE in preventing the cisplatin induced DNA damage and leading apoptosis. However, the expression of p53 persisted at low level even at 45th day, indicating the failure of APE in completely preventing the cisplatin apoptotic process. Perusal of literature did not reveal any report on the effect of *Andrographis paniculata* in expression of p53 in cisplatin toxicity. The study suggested that *Andrographis paniculata* has anti-apoptotic effect probably through its antioxidant activity and quenching of free radicals that cause DNA damage.

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