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Patulin causes DNA damage leading to cell cycle arrest and apoptosis through modulation of Bax, p^{53} and $p^{21/WAF1}$ proteins in skin of mice

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ABSTRACT

Patulin (PAT), a mycotoxin found in apples, grapes, oranges, pear and peaches, is a potent genotoxic compound. WHO has highlighted the need for the study of cutaneous toxicity of PAT as manual labour is employed during pre and post harvest stages, thereby causing direct exposure to skin. In the present study cutaneous toxicity of PAT was evaluated following topical application to Swiss Albino mice. Dermal exposure of PAT, to mice for 4 h resulted in a dose (40–160 μg/animal) and time (up to 6 h) dependent enhancement of ornithine decarboxylase (ODC), a marker enzyme of cell proliferation. The ODC activity was found to be normal after 12 and 24 h treatment of patulin. Topical application of PAT (160 μg/100 μl acetone) for 24–72 h caused (a) DNA damage in skin cells showing significant increase (34–63%) in olive tail moment, a parameter of Comet assay (b) significant G 1 and S-phase arrest along with induction of apoptosis (2.8–10 folds) as shown by annexin V and PI staining assay through flow cytometer. Moreover PAT leads to over expression of $p^{21/WAF1}$ (3.6–3.9 fold), pro apoptotic protein Bax (1.3–2.6) and tumor suppressor wild type p^{53} (2.8–3.9 fold) protein. It was also shown that PAT induced apoptosis was mediated through mitochondrial intrinsic pathway as revealed through the release of cytochrome *C* protein in cytosol leading to enhancement of caspase-3 activity in skin cells of mice. These results suggest that PAT has a potential to induce DNA damage leading to p^{53} mediated cell cycle arrest along with intrinsic pathway mediated apoptosis that may also be correlated with enhanced polyamine production as evident by induction of ODC activity, which may have dermal toxicological implications.

Introduction

Patulin (4-hydroxy-4H-furo {2, 3-C} pyron-2 {6H}-1) (clavacin; PAT; Fig. 1), is a mycotoxin produced by several species of *Penicillium*, *Aspergillus* and *Byssochlamys* (Steiman et al., 1989). It is a common contaminant of ripe apples, used for the production of juice concentrates owing to its solubility in water (Harrison, 1989). PAT has also been determined in pears, apricots, peaches, tomatoes, grapes and oranges and in products derived from these fruits (Prieta et al., 1993; Harrison, 1989). Considering the toxicity of patulin, several countries round the globe have set a maximum permissible level of patulin as 50 ppb in foodstuffs (Codex, 2003; USFDA, 2004; EU, 2003).

PAT has been classified as group-3 carcinogen (IARC, 1987). Several organs including kidney, liver, intestinal tissue and immune system have been found to be affected by *in vivo* administration of PAT (Speijers et al., 1988; Wichmann et al., 2002). Apart from the acute toxic effects, PAT is reported to be teratogenic and carcinogenic in certain experimental animals (Ciegler et al., 1976; Osswald et al., 1978). Few studies also reveal PAT induced DNA damage, chromosome aberration, and micronuclei formation in mammalian cells (Alves et al., 2000; Thust et al., 1982; Liu et al., 2003). A previous study shows a rapid and persistent activation of extracellular signal regulated protein kinases 1 and 2 (ERK 1/2) by PAT exposure in certain human cell lines like embryonic kidney (HEK293) cells, peripheral blood mononuclear cells (PBMCs), and Madin–Darby canine kidney (MDCK) cells (Wu et al., 2005). Findings of similar group have also revealed that PAT leads to rapid activation of two more major mitogen-activated protein kinases (MAPKs), p38 kinase and c-Jun N-terminal kinase (JNK) in HEK293 cells (Liu et al., 2006), however only p38 kinase signaling pathway contributes to PAT induced cell death. PAT being electrophilic in nature exerts cytotoxic and immunotoxic effects mainly through binding with sulphydryl groups, proteins and amino acids in the plasma membrane (Riley and Showker, 1991). PAT has been shown to cause effects on cell-cycle distribution, which is responsible for over expression of a functional p^{53} protein in V79 cell lines and primary human skin fibroblasts (Lehmann et al., 2003).

The disruption of the equilibrium between cell proliferation and cell death is considered to be an early and important event in carcinogenic process (Di Giovanni, 1992). Recent evidence indicates

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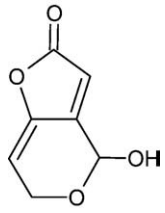

Fig. 1. Structure of patulin.

that polyamines, cell proliferation, and apoptosis are tightly connected in a quite complex interplay (Tantini et al., 2006). It appears that polyamines are Janus-faced regulators of cellular fate, promoting either cell proliferation or cell death, depending on the cell type as well as on the environmental signals (Thomas and Thomas, 2001). In several cell types high levels of polyamines may directly cause cell death (Poulin et al., 1995; Stefanelli et al. 1998; Erez et al., 2002). Polyamine levels within the cells are regulated and modulated by the key enzymes that control polyamine biosynthesis particularly ornithine decarboxylase activity (ODC) that rapidly responds to several stimuli, which is mainly linked to cell growth and/or toxicity (Wallace et al., 2003; Thomas and Thomas, 2001). Furthermore, a recent study reveals that elevated

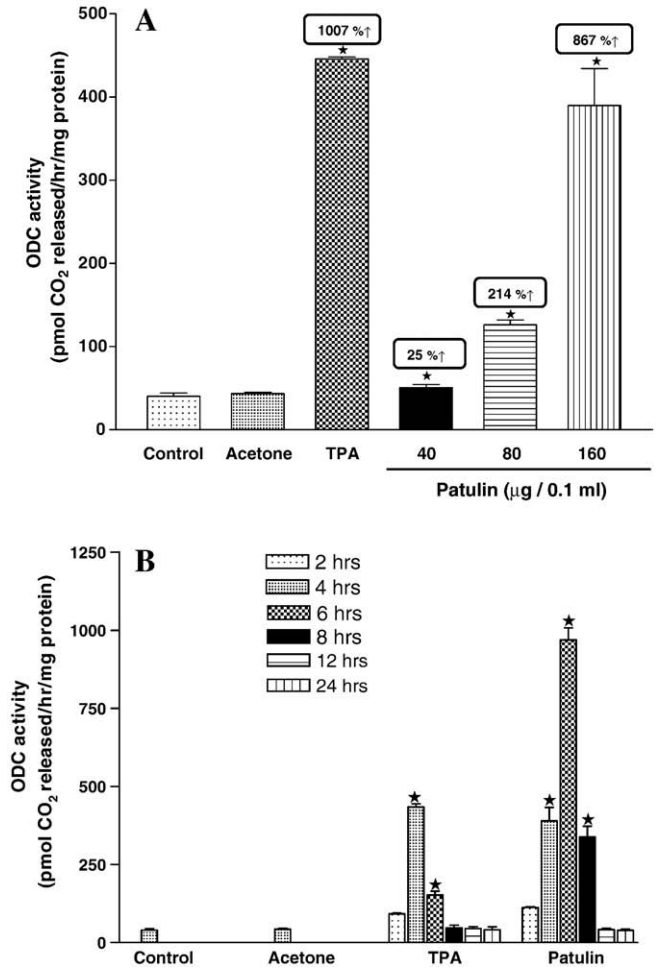

Fig. 2. Effect of topical application of patulin on epidermal ODC activity of mice. (A) Dose dependent effect of topical application of patulin and TPA on epidermal ODC activity of mice exposed for 4 h. (B) Time dependent effect of patulin on epidermal ODC activity of mice exposed for 2–24 h. The dose regimen and treatment protocols are described in Materials and methods. The dose for time dependent effect of patulin was 160 µg/0.1 ml. The skin ODC activity was measured in cytosolic fraction using [^{14}C] ornithine as substrate. Each value represents the mean±SE of three animals. *P<0.05, significant with respect to control.

Table 1
DNA damaging potential of patulin in skin cells of mice

Group	Tail moment (arbitrary units)	Tail DNA (%)	Tail length (µM)
Control	5.03±0.25	5.98±1.14	48.22±1.24
Patulin (6 h) (160 µg/mouse)	5.14±0.08	6.04±0.08	50.14±0.08
Patulin (12 h) (160 µg/mouse)	5.09±0.42	4.14±0.08	51.27±0.29
Patulin (24 h) (160 µg/mouse)	6.72±0.32* (34%↑)	7.89±0.36* (32%↑)	64.93±1.52* (35%↑)
Patulin (48 h) (160 µg/mouse)	7.88±0.92* (57%↑)	10.23±1.05* (71%↑)	89.67±3.04* (86%↑)
Patulin (72 h) (160 µg/mouse)	8.19±0.59* (63%↑)	15.03±1.48* (151%↑)	108.20±3.15* (124%↑)
Benzo(a)pyrene (160 µg/mouse)	10.26±1.01* (104%↑)	22.92±0.32* (283%↑)	110.40±2.91* (128%↑)

Data represent mean±S.E. of 3 animals in each group.
Values in parenthesis indicate percent increase (↑).
Details of treatment schedule and processing of cells are mentioned in Materials and methods.
* P<0.05, significant when compared to control.

ODC activity and increased biosynthesis of polyamines serve as a novel stimulus to induce the ataxia telangiectasia mutated (ATM)-DNA damage signaling pathway and cell death in normal keratinocytes (Wei et al., 2008) The tumor suppressor gene p^{53} mediates activation of programmed cell death, in part by up-regulation of mitochondrial Bax expression (Miyashita et al., 1994). Bax is a key component for apoptosis through mitochondrial stress (Wei et al., 2001). Bax forms oligomers and translocates from the cytosol to the mitochondrial membrane (Jurgensmeier et al., 1998). It increases the membranes permeability through interactions with the pore proteins on the mitochondrial membrane, which leads to the release of cytochrome *C* from mitochondria, and activation of caspase 3, a notable effector in apoptosis which is a convergence point for two different caspase dependent apoptosis pathway (Narita et al., 1998).

According to WHO guidelines, humans and animals may be exposed to mycotoxins through ingestion, inhalation or skin contact (WHO, 1998). Limited knowledge is available regarding epidermal carcinogenesis of mycotoxins and WHO has clearly highlighted the need for toxicological evaluation of mycotoxins through dermal exposure (Anonymous, 1990). This is an important aspect from the point of view of developing countries in tropics including India where manual labour is employed during pre- and post-harvest stages in agriculture thus indicating a probable cause of exposure through dermal route. In this regard our prior studies have revealed cutaneous carcinogenic implications of a mycotoxin, Aflatoxin-B1 (AFB1) through dermal exposure and in a sequel study it is shown that extract of Ocimum sanctum leaf may cause

Table 2
Different phases of skin cells of mice topically treated with patulin

Group	Cell cycle phase		
	G0/G1	S	G2/M
Control	35.3±0.4	16.8±0.6	12.3±2.3
Patulin (24 h) (160 µg/mouse)	46.0±0.9* 30%↑	17.5±2.4	11.0±0.2
Patulin (48 h) (160 µg/mouse)	39.7±0.5* 13%↑	23.0±1.2* 37%↑	10.0±1.4
Patulin (72 h) (160 µg/mouse)	34.5±1.0	34.0±1.9* 103%↑	11.7±1.6
Benzo(a)pyrene (160 µg/mouse)	40.1±0.7* 14%↑	42.0±0.7* 147%↑	10.8±0.3

Data represent mean±S.E. of 3 animals per group.
Values in parenthesis indicate percent increase (↑) or decrease (↓).
Details of treatment schedule and processing of cells are mentioned in Materials and methods.
* P<0.05, significant when compared to control.