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Original Contribution

Size restricted silymarin suspension evokes integrated adaptive response against acute hypoxia exposure in rat lung

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ABSTRACT

Despite its extraordinary antioxidant capacity, the clinical usage of silymarin has remained restricted to amelioration of hepatic pathology. Perhaps its low bioavailability and uneven bio-distribution, owing to its poor aqueous solubility, are two main causes that have dampened the clinical applicability and scope of this preparation. We took these two challenges and suggested an unexplored application of silymarin. Apart from liver, two of the most susceptible vital organs at the highest risk of oxidative stress are brain and lung, especially during reduced oxygen saturation (hypoxia) at anatomical level. Hypoxia causes excess generation of radicals primarily in the lungs as it is the first organ at the interphase of atmosphere and organism making it the most prone and vulnerable to oxidative stress and the first responder against hypobaric hypoxia. As our first objective, we improved the silymarin formulation by restricting its size to the lower threshold and then successfully tested the prophylactic and therapeutic action in rat lung challenged with simulated hypobaric hypoxia. After dose optimization, we observed that 50 mg/kg BW silymarin as size restricted and homogenous aqueous suspension successfully minimized the reactive oxygen species and augmented the antioxidant defense by significant upregulation of catalase and superoxide dismutase and reduced glutathione. Moreover, the well-established hypoxia markers and proteins related to hypoxia adaptability, hif1a and VEGF were differentially regulated conferring significant reduction in the inflammation caused by hypobaric hypoxia. We therefore report,the unexplored potential benefits of silymarin for preventing high altitude associated pathophysiology further paving its road to clinical trials.

1. Introduction

Reduced oxygen bioavailability at high altitudes or hypobaric hypoxia is a well-known physiological challenge and the scale of problem may be linked to the fact that nearly 24 percent of the global land area is mountain and home for around 140 million people [1]. The severity and ill effects are worsened during rapid ascent to altitude from sea level. At cellular level this physiological challenge is evidently concomitant with elevated reactive oxygen species and downstream effects of oxidative stress caused thereof. Lowered oxygen concentration at cellular level results in accumulation of reduction equivalents such as NADH which cross donate their electrons resulting in the generation of free radicals [2–4]. Apart from the biochemical alteration caused by radical or non-radical oxidants, radical induced protein modifications and localized compensatory immune response are the major ill-effects of hypobaric hypoxia leading to clinical pathology often referred to as acute mountain sickness [5–7]. Hypoxia induced oxidative stress becomes more pronounced during the rapid ascent of individuals to high altitude [8]. Pre-supplementation with plant based-polyphenols due to their prophylactic benefits and nontoxic nature is one of the most preferred modes of intervention to defend against ill effects of hypoxia [9–11].

Several studies have shown the use of various phyto-extracts to minimize the radical generation in hypoxia. Phyto-extracts from sea buckthorn and *Withania somnifera*; huperzine A, quercetin, ascorbic acid, α-tocopherol, phyto-extracts, melatonin, vitamin E, carotene, poly-phenols, Acetyl-L-carnitine (ALCAR), alpha-ketoglutaric acid and 5-hydroxymethyl-2-furfural (5-HMF) were shown to limit hypoxia-induced oxidative stress [12–15]. Neuronal cells pre-incubated with 50 or 100 mM of the phenolic antioxidant 3,3′,5,5′-tetra-t-butyl-biphenyl-4,4′-diol (BP) prior to hypoxia- reperfusion (H/R) injury showed reduced ROS accumulation, limited endogenous gene response and cell death [16,17]. However, these

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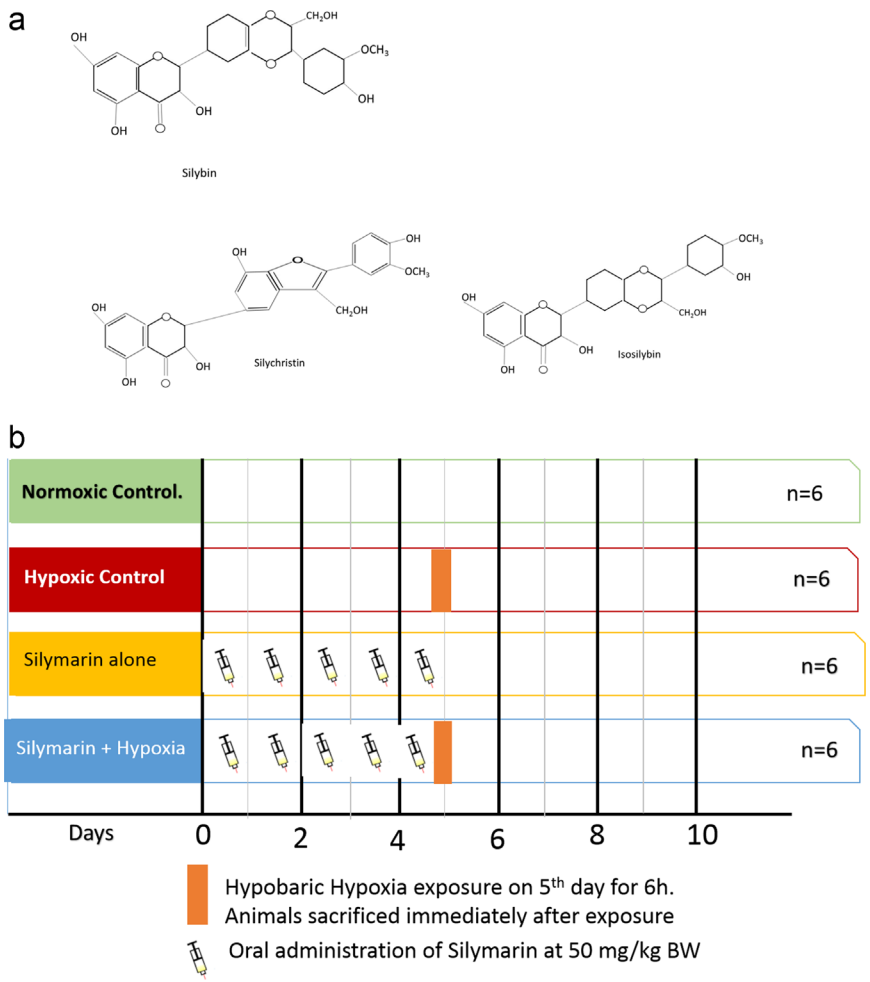

Fig. 1. Active polyphenols of silymarin and experiment design. (a) Active polyphenols present in silymarin include Silybin, Isosilybin, Silychristin were observed. (b) Male SD rats weighing about 200 g (8 weeks old) were divided into four groups with n=6 for each group - Normoxic control, Hypoxic control (received only normal saline), silymarin pre-treated group exposed to hypobaric hypoxia (25,000 ft for 6 h) and silymarin pre-treated group kept in normoxic conditions. The hypoxic controls and silymarin pre-treated hypoxic animals were given either normal saline or silymarin at 50 mg/kg BW orally for 5 days. Dosing was done at intervals of 24 h. The silymarin treated normoxic animals also underwent the same dosing regimen. On the 5th day, 1 h post oral silymarin administration, the hypoxic controls and the silymarin treated hypoxic group underwent 6 h of hypobaric hypoxia exposure at a simulated altitude of 25,000 ft.

extracts need to be administered in very high doses (usually g/kg BW) or in doses repeated for a long time-span [18,19]. The challenges were low solubility and bioavailability of these products which usually mask their benefits. Another challenge was hepatic-metabolism of any polyphenol and its excretion thereby reducing its efficacy [19]. Due to these limitations many of the polyphenol supplementation studies fail to qualify in clinical trials and newer molecules with improved features are being continuously sought.

Historically, milk thistle (*Silybum marianum* sp.) plant extract has been used as a prophylactic and herbal therapeutic in various liver conditions like liver cirrhosis, hepatitis and necrosis [20]. Recently, some of the clinical studies have also been conducted, an integrated metadata analysis of clinical trials was provided by Tamayo et al., which highlights some of the clinical usage of silymarin [21]. Several studies have also confirmed the active principles of the extract which is now recognized as silymarin. It is a mixture of flavonolignans, namely, Silybin, Isosilybin, and Silychristin (Fig. 1a), among which Silybin is the chief active constituent [22,23]. For over a decade, the benefits of silymarin have been explored beyond hepatotoxicity and tested to a great extent in various disease models. Some of the key research revealed its anticancer and chemoprotective behavior [24]. Hypocholesterolemic properties conferring cardioprotection have also been evaluated in vivo [25]. Wang et al. have shown the neuroactive and neuroprotective activities of silymarin [26]. Moreover, the scope of its applications has been further extended to several other organ systems such as treatment of pancreatic problems [27], balancing glycemia [28], treatment and prevention of dermal disorders, including use in cosmetic preparations [29]. Additionally, prevention and treatment of prostate hyperplasia involving adenocarcinoma is a recent silymarin application, which could successfully reach phase II trials [30].

Pharmacokinetic studies have shown that silymarin is absorbed by the oral route and that it distributes into the alimentary tract (liver, stomach, intestine, and pancreas). It is mainly excreted as metabolites in the bile, and is subject to enterohepatic circulation [31,32]. Toxicity is almost negligible, the oral 50% lethal dose being 10,000 mg/kg in rats and the maximum tolerated dose being 300 mg/kg in dogs [33–35]. Moreover, silymarin is devoid of embryotoxic potential [36]. In another study by Baer-Dubowska, silymarin was also found to inhibit the hepatic cytochrome P450 (CYP) detoxification system (phase I metabolism), which indicates its abilities to evade the routine drug metabolic processes and have increased persistence [37].

Comprehension of silymarin's myriad potential mechanisms and downstream effects catering the anti-oxidant defenses within an organism is still nascent, however a landmark study by Gazak et al. has demonstrated the importance of hydroxyl group