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Full Length Research Paper

The role of the yeast as probiotic in protection against liver injury

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Saccharomyces cerevisiae **is a well known yeast used safely from ancient times in many biotechnological applications. Nowadays, there is an increasing interest in using yeast as probiotic. Dimethyl nitrosamine (DMN) has been used to induce liver fibrosis in rats. Yeast has been used side by side with the DMN to evaluate the role of its use as a probiotic in the protection against liver fibrosis. Six groups of rats have been used to represent negative and positive controls and other four groups which have been treated by DMN for two weeks and killed after 35 or 60 days. Two of the DMN treatment groups have been fed by yeast from the first 35 to 60 days, respectively. The results show that yeast has no side effect on each of glutamate-oxaloacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT) and alkaline phosphatase (ALP) enzyme activities. DMN showed a significant effect on those enzymes. Feeding with yeast for 35 days showed a minor improvement, while feeding them for 60 days showed a significant improvement. Moreover, the expressions of interleukin 1-alpha (IL-1α) and interleukin-7 receptor (IL-7r) have been evaluated using reverse transcription polymerase chain reaction (RT-PCR). The study includes also the analysis of liver histopathology. This study shows the importance of using yeasts as probiotics in the protection against liver injury.**

Key words: Yeast, *Saccharomyces cerevisiae*, probiotic, dimethyl nitrosamine, IL-7r, IL-1α.

INTRODUCTION

The term "probiotic" means "for life". It generally refers to concentrated supplements of beneficial bacteria similar to those that occur naturally in the intestinal tract. Most probiotic supplements are formulated to survive the digestive process and the highly acidic conditions in the stomach (Heilpern and Szilagyi, 2008; Heimbach, 2008; Macfarlane and Cummings, 1999a, b; Marchand and Vandenplas, 2000). Yeast are used in many industrial, medicinal and pharmaceutical applications (Kumura et al., 2004; Brophy et al., 2008; Buts, 2005; Cohen, 2007; Gerasimidis et al., 2008; Huebner et al., 2008; Rosen et al., 2007). In recent years, showing an increasing interest in using yeast as probiotic has been observed (Beverage, 2006; van der Aa Kule et al., 2005; Sullivan and Nord, 2003). *Saccharomyces cerevisiae var boulordii* has been used widely for treating a range of diarrheal disorders (van der Aa Kule et al., 2005; Czervoka and Rampal, 2002). Many other yeasts species have been described such as *Debaryomyces hansenii, Kluy marxianus,*

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Abbreviations: DMN, Dimethyl nitrosamine; **GOT,** glutamate-oxaloacetate-transaminase; **GPT,** glutamate-pyruvate-transaminase; **ALP,** alkaline phosphatase; **RT-PCR,** reverse transcription polymerase chain reaction; **IL-1α** interleukin 1-alpha; **IL-7r,** interleukin-7 receptor; **LSD,** least significant difference.

Yarrowia lipolytica, Idastrandia orientalis, *Pichia farinose* and *Pichia anomala* (Beverages, 2006). Different kinds of probiotic have been examined as a potential source for cancer treatment (Chen et al., 2009). In this study, we investigated the role of *S. cerevisiae* in rats liver protection against fibrosis during the treatment with dimethyl nitrosamine (DMN) as a mutagen.

MATERIALS AND METHODS

Preparation of *Saccharomyces cerevisiae*

S. cerevisiae strain bought from local market as dry granules has been used in this study. It was prepared as a semi wet paste. The strain was subjected to activation before its use by mixing it with 5 ml of 2% glucose for each 25 g under aseptic conditions, and left at 37°C till suitable dryness which enabled better mixing with the animal food. The final yeast amount eaten by each rat/day was 500 mg/kg/day.

Animals

Adult male Wistar albino rats, ageing approximately three months and ranging in weight from 150 ± 10 g, were obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The animals were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water. The protocol of this study has been followed by the instruction of the Research Ethics Committee of College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Chemicals

Chemicals used in this research include: glutamate-oxaloacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT) and alkaline phosphatase (ALP) purchased from USA-Randox Laboratories®; DMN purchased from Sigma Chemical®, St. Louis, MO, USA; TRIzol reagent purchased from Invitrogen® (Grand Island, NY); High-Capacity cDNA reverse transcription kits and SYBR Green PCR Master Mix purchased from Applied Biosystems® (Foster City, CA, USA) and primers purchased from MSAM International Est®, Riyadh, Kingdom of Saudi Arabia.

Experimental design

Six groups of rats have been investigated and represented: group 1, 10 rats as negative control (without any treatment only food for 60 days); group 2, 10 rats fed as the above group but yeast was added as a positive control; group 3, 10 rats injected intraperitoneally (IP) with DMN for two weeks during the feeding with food containing yeast for 35 days; group 4, 10 rats injected IP with DMN for two weeks during the feeding period, with food containing yeast for 60 days; group 5, 10 rats injected IP with DMN for two weeks during the feeding period, with food only for 35 days and group 6, 10 rats injected IP with DMN for two weeks during the feeding period, with food only for 60 days. The amount of DMN was 100 μM (1 μg/kg). The rats were left after last feeding overnight without feeding and were weighed before sacrifice. At the end of the treatment protocol, rats were been subjected to complete anesthesia using diethyl ether. Blood sample from each rat was collected. The abdominal cavities were immediately opened and the liver was excised, weighed, and then washed with cold 0.1 M phosphate buffer, pH 7.4.

Biochemical analysis (GOT, GPT and ALP)

The blood sample were collected from each rat in sterilize eppendorf tube as above, stored in room temperature (20°C) for 2 min, then centrifuge at 2000 rpm. GOT, GPT and ALP activities were determined spectrophotometrically with the aid of USA-Randox® kit. The increase in the absorbance against time was measured at different time interval and the best slope was determined for each sample. The activity from each slope was determined against standard curve for each of GOT, GPT and ALP.

RNA extraction and cDNA synthesis

Total RNA from the liver tissue homogenate was isolated using TRIzol reagent (Invitrogen®). The isolation method was performed according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm; the RNA quality was determined by measuring the 260/280 ratio. The cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems®) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mixture of 2.0 μl of 10× reverse transcriptase buffer, 0.8 μl of 25× dNTP mix (l00 mM), 2.0 μl of l0× reverse transcriptase random primers, 1.0 μl of MultiScribe reverse transcriptase, and 3.2 μl of nuclease-free water. The final reaction mixture was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 s, and finally cooled to 4°C.

Quantification of mRNA expression by reverse transcription polymerase chain reaction (RT-PCR)

Quantitative analysis of mRNA expression of target genes was performed by RT-PCR through subjecting the resulting cDNA from the above preparation to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems®). The 25 μl reaction mixture contained 0.1 μl of 10 μM forward primer and 0.1 μl of 10 μM reverse primer (40 μM final concentration of each primer), 12.5 μl of SYBR Green Universal Mastermix, 11.05 μl of nuclease-free water, and 1.25 μl of cDNA sample. The primers used in the current study were chosen from previously published studies as listed in Table 1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The real-time PCR data was analyzed using the relative gene expression (that is, $\Delta\Delta$CT) method, as described in Applied Biosystems® User Bulletin no. 2. Briefly, the data were presented as the fold change in gene expression normalized to the endogenous reference gene (B actin) and relative to a calibrator.

Histological studies

At the end of treatment, both control and experimental rats were euthanized using diethyl ether. The livers were collected from all the groups, fixed in 10% formalin in saline, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in molten paraplast at 58 to 62°C. Thin sions (4 to 5 mm) were cut using microtome, and stained with hematoxylin and eosin, and then examined using a light microscope. Histological changes in the liver were scored for identification of organ architecture and presence of edema, inflammatory cell infiltrate, hemorrhage and ulceration. The

Table 1. Forward and reverse primers used in this study.

Gene	Forward primer	Reverse primer	References
IL-1α	GGAGGCCATAGCCCATGATTTAG	TATTCTGGAAGCTGTGTAGGTGCTG	Xia et al. (2010)
IL-7r	SCAGAGAAGGTGACGGAAATAGGT	ACTGGAGAGGAAATGGTTGAGG	Xia et al. (2010)
B actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG	Watson et al. (1992)

Table 2. Gene expression for both IL-1α and IL-7 R in liver mice for different treatment.

Gene		Control	Yeast	Yeast + DMN 35 days	Yeast + DMN 60 days	DMN 35 days	DMN 60 days
IL-1α	RQ	1	0.800564	0.87492	2.587842	1.063101	4.653063
	SE	0.04	0.14	0.08	0.09	0.05	0.3
	RQ	1	0.540631	0.567112	0.279881	0.751756	1.971113
IL-7r	SE	0.07	0.02	0.04	0.03	0.033	0.06

Table 3. Fisher's LSD for GOT, GPT and ALP.

Contrast	GOT		GPT		ALP	
	Significant	Difference	Significant	Difference	Significant	Difference
Control-DMN 30 days	*	-87.6	*	-47.4	*	-224.8
Control-DMN 60 days	*	-110.4	*	-57.8	*	-259.4
Control-DMN-Yeast 35 days	*	-84.8	*	-18.4	*	-84.4
Control-DMN-Yeast 60days	*	-24		0.4		3.8
Control-Yeast		2		1		5.6
DMN 30 days-DMN 60 days	*	-22.8	*	-10.4		-34.6
DMN 30 days-DMN-Yeast 35 days		2.8	*	29	*	140.4
DMN 30 days-DMN-Yeast 60 days	*	63.6	*	47.8	*	228.6
DMN 30 days-Yeast	*	89.6	*	48.4	*	230.4
DMN 60 days-DMN-Yeast 35 days	*	25.6	*	39.4	*	175
DMN 60 days-DMN Yeast 60 days	*	86.4	*	58.2	*	263.2
DMN 60 days-Yeast	*	112.4	*	58.8	*	265
DMN-Yeast 35 days-DMN-Yeast 60 days	*	60.8	*	18.8	*	88.2
DMN-Yeast 35 days-Yeast	*	86.8	*	19.4	*	90
DMN-Yeast 60 days-Yeast	*	26		0.6		1.8

+/- Limits GOT, 12.518; GPT, 8.74179; ALP, 36.1444.

slides were prepared to be ready for examination in College of Science, Zoology Department, King Saud University, Riyadh, Saudi Arabia. For examination in the light microscope, briefly, a field containing a portal vein approximately 100 μm in diameter in its centre was selected at a magnification of 200×. Digitalized images of the field and five random fields of the same size were captured for computer analysis by a digital camera. The average of the five fields was calculated for assessment of the degree of fibrosis in each case.

Statistical analysis

The data from each of GOT, GPT and ALP, as well as from RT-PCR of the gene expression of interleukin 1-alpha (IL-1α) and interleukin-7 receptor (IL-7r) was subjected to analysis using analysis of variance (ANOVA) test to determine the P-value for the different used variables. The Fisher's least significant difference (LSD) test was used (within this method, 5.0% risk of calling each pair of means significantly different when the actual difference equal 0) to find the significant differences between each two variables. The results are been summarized in Tables 2, 3 and 4.

RESULTS AND DISCUSSION

From Tables 3 and 4 and Figures 1 to 3, it is clear that positive and negative controls are nearly same which indicate that yeast has no negative effect on the GOT liver function and can be used safely. In case of treatment with DMN for two weeks, GOT activity increased significantly after 35 days and a further

Table 4. Fisher's LSD for IL-1α and IL-7r.

Contrast	IIR		IIL	
	Significant	Difference	Significant	Difference
Control-DMN 35 days	*	-1.13812	*	-0.36041
Control-DMN 60 days	*	-0.31188	*	-0.37055
Control-Yeast	*	-0.60878	*	-0.84901
Control-Yeast-DMN 35 days	*	-0.21027	*	-0.45532
Control-Yeast-DMN 60 days	*	-1.9529	*	-0.7347
DMN 35 days-DMN 60 days	*	0.826238	*	-0.01014
DMN 35 days-Yeast	*	0.529344	*	-0.4886
DMN 35 days-Yeast-DMN 35 days	*	0.927854	*	-0.0949
DMN 35 days-Yeast-DMN 60 days	*	-0.81478	*	-0.37429
DMN 60 days-Yeast	*	-0.29689	*	-0.47846
DMN 60-Yeast-DMN 35 days	*	0.101615	*	-0.08476
DMN 60 days-Yeast-DMN 60 days	*	-1.64102	*	-0.36415
Yeast-Yeast- DMN 35 days	*	*0.39851*	*	0.393694
Yeast-Yeast-DMN 60 days	*	-1.34413	*	0.114313
Yeast-DMN 35 days-Yeast-DMN 60 days	*	-1.74264	*	-0.27938

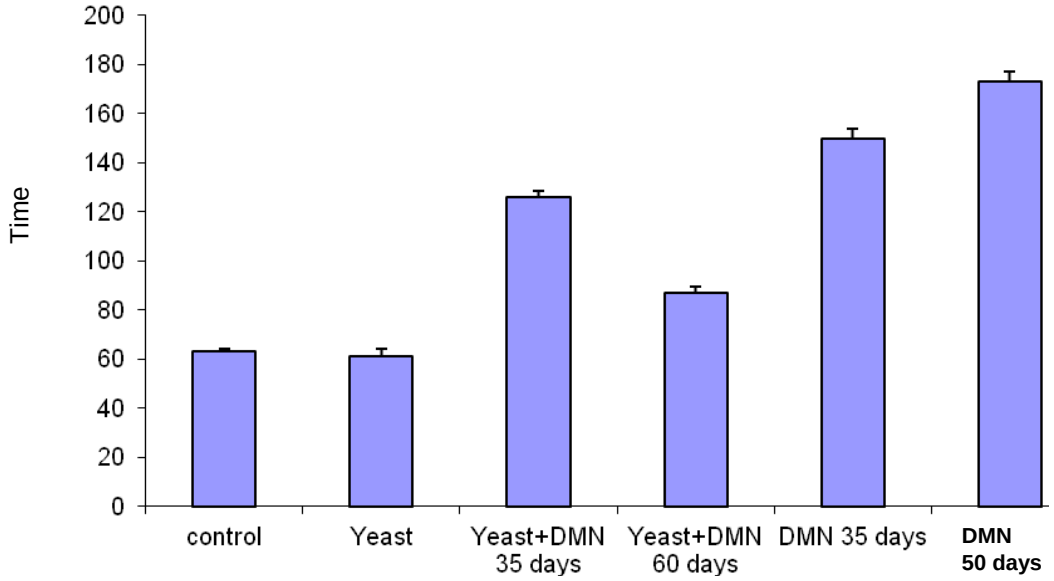

Figure 1. Histogram showing biochemical analysis for GOT.

increase was shown after 60 days, which prove that the liver functions was been impaired by the mutagen DMN. This change increased by the time after treatment by DMN. This is a clear induction about that liver injury or fibrosis has happened. In case of using yeast during the treatment with DMN for 35 days, negligible improvement in the liver function was shown, and the amount of GOT was still high. However, treatments with yeast for 60 days showed a significant improvement in the liver function and the level of GOT decreased significantly. The same results were obtained in case of GPT and ALP.

Recently Xia et al. (2010) demonstrated changes that happened in both of IL-1α as well as IL-7r. This change happened during hepatocellular carcinoma, developed due to liver injury. These two markers have been used in our study to investigate their expression during the induction of liver injury. DMN has been used in liver injury induction. Interleukin-7 receptor is a protein found in the surface of the cell (Huebner et al., 2008). Interleukin-7 receptor has been reported by different authors to be associated with different kind of diseases including liver injury, T-cell acute lymphoblastic leukemia and multiple sclerosis and rheumatoid arthritis (Nicklin et al., 1994; Watson and Miller, 1995; Al-Rawi et al., 2003). IL-1α is

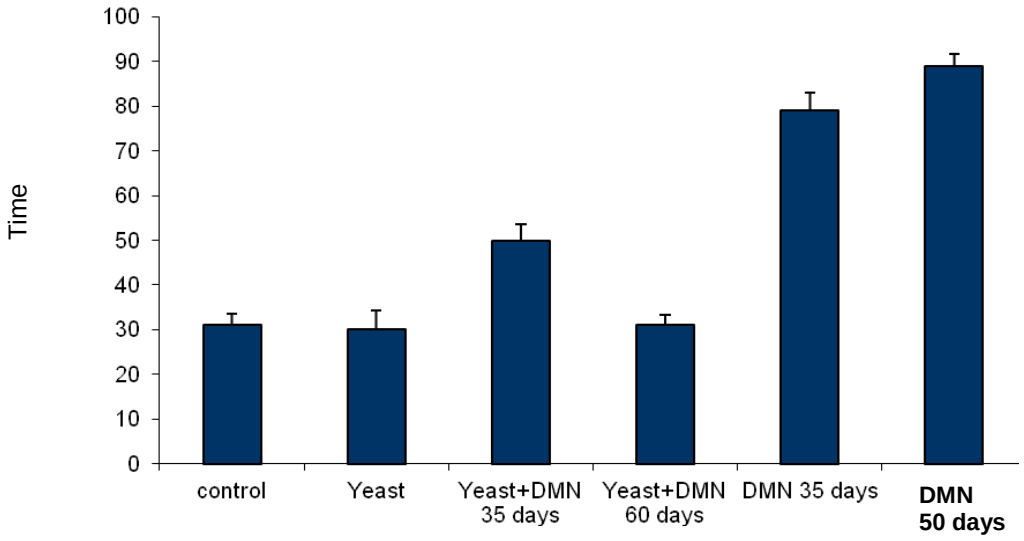

Figure 2. Histogram showing biochemical analysis for GPT.

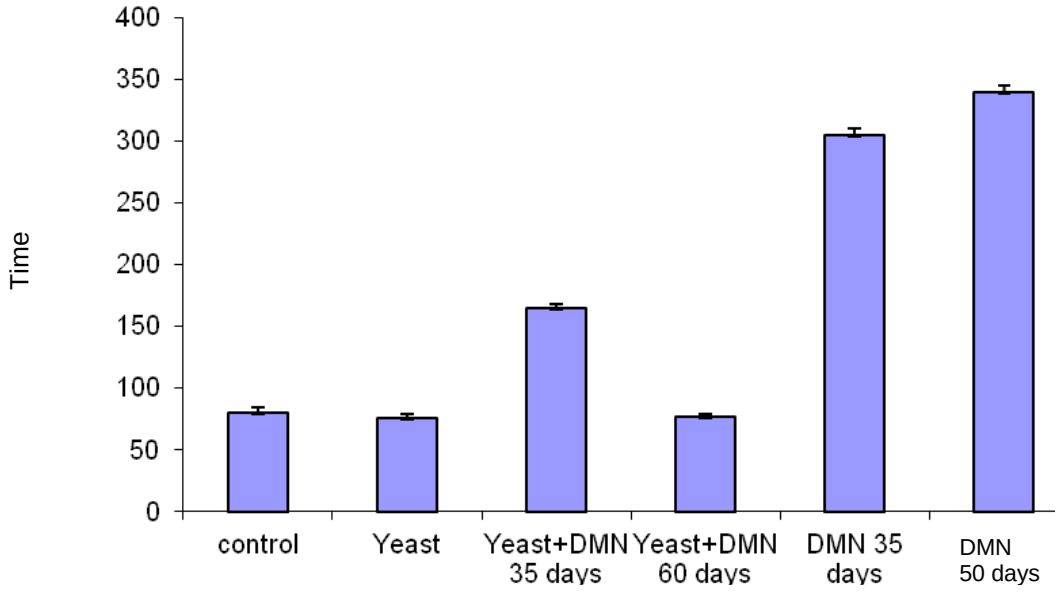

Figure 3. Histogram showing biochemical analysis for alkaline phosphatase.

the protein encoded by the *IL1a* gene (Marchand et al., 2000). As a cytokine, it has many important activities which have been reported to be influenced when physiological changes existed, as well as in the hematopoietic and liver injury (Bankers-Fulbright et al., 1996; Watson and Miller, 1995). Although there are many interactions between IL-1α and the other cytokines, the most consistent and most clinically relevant is its synergism with tumor necrosis factor (TNF) (van Der, 2005). In this study, as shown in Table 2, it can be observed that the normal condition of IL-1α and IL-7r expressions in group 1 shows a low level of the expressions of both genes. Also, their expressions amounts in general, have been increased independently from the types of the inducer. Each of yeast and DMN or both of them had increased expression of both genes. This spots the roles of IL-1α and IL-7r in sensing and interacting with any abnormal conditions faced by the cells. However, the data tells us that there are two types of responses during various treatments; one is positively affected and the other is negatively affected. The positive affect is mainly due to yeast, where the cells, still normal, and the excesses in our genes due to abnormal conditions, could be maintained, while cells itself is still

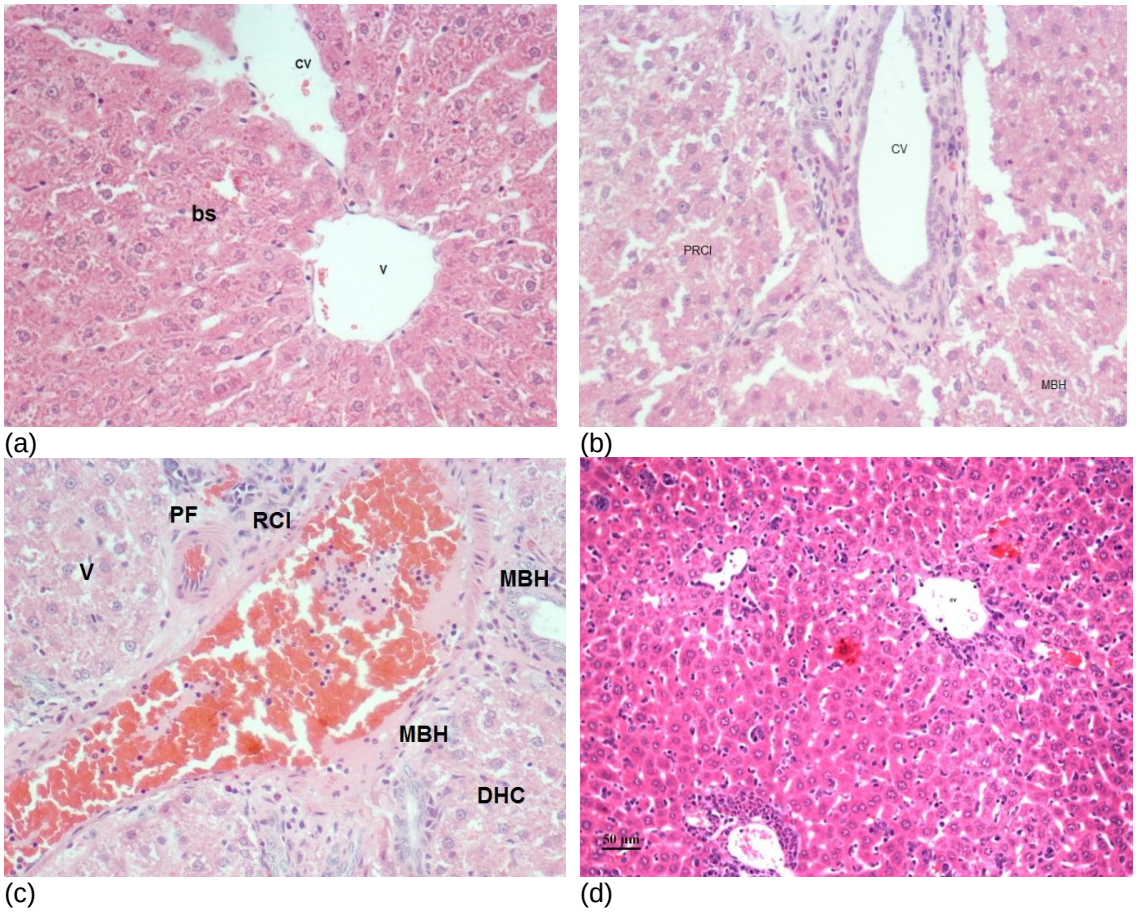

Figure 4. (a) Histology of normal control rat liver; (b) pronounced histopathological abnormalities seen in rats treated with DMN 35 days; (c) DMN treated (60 days) rat liver showing higher tendency for liver fibrosis manifested by the presence of many spots of focal collected cellular granulomatous lesions which showed focal collection of inflammatory cells and loss of hepatic tissue structural pattern; (d) treated with only yeast. CV, Central vein; BS, blood sinusoids; DHC, degenerated hepatic cord ; PF, periportal fibrosis; RCI, periportal round cell infiltration; MBH, massive breakdown hepatocytes; V, vacuole; RCI, round cell infiltration.

active and viable. This can be concluded from both gene expressions by comparing groups 3 and 4 with groups 5 and 6. Particularly in group 4, both genes showed high expressions. However, in case of group 5 and 6, the expression in both genes came down. This agrees with the data from the histopathology.

Yeast maintains the cells viability and as a result, maintains both gene expressions. From the expression of IL-1α in group 2 which shows the highest expression, it is clear that yeast is a perfect inducer for that gene and for some extent to the IL-7r. The decrease in the expression in case of IL-7r, can be explained due to the fact that, the cells sensed that the yeast is not that harmful an agent, especially if compared with group 5, where DMN existed alone. It is clear that yeast, even though it is an inducer, it is still a friendly inducer for both genes while cells are still viable. DMN is also an inducer but had negative effect on the cell viability which can be concluded for the expression of both genes in group 6. Time is a critical factor. Comparing time can reveal significant conclusions about what is going on. IL-1α has been linked to the activation of TNFα or at least, there is a synergistic interaction between both of them, which might reveal that IL-1α is a modulator for the endogenous antioxidant systems, which is a strong protector against oxidants and free-radicals, as well as mutagens (Amro, 2010; Dinarello, 2001).

In case of the data obtained from the histopathology examination, they are in agreement with all the above results, and yeast shows a significant ability to maintain the liver. Light microscopic observation revealed that the control, negative control and the groups treated with yeast showed normal large polygonal cells with prominent round nuclei and eosinophilic cytoplasm, and few spaced hepatic sinusoids arranged in between the hepatic cords with fine arrangement of Kupffer cells (Figures 4a and d). In contrast, groups receiving only DMN for 35 days showed massive hepatotoxicity.

However, DMN alone for 60 days exhibited increased hepatotoxicity in comparison to other treatment. The most

pronounced histopathological abnormalities observed in rats treated with DMN are dissolution of hepatic cords, which appeared as empty vacuoles aligned by strands of necrotic hepatocytes (Figures 4b and c). The hepatic tissues showed the presence of dense focal inflammatory cells or necrotic tissues. 35 and 60 days treatment of DMN alone resulted in common histopathological alterations, including perivascular round cell infiltration, associated with membrane changes of endothelial lining cells manifesting periportal fibrosis, marked degeneration of hepatic cords, increased incidence of vacuolar degeneration and apoptotic cell death. DMN treatment showed higher tendency for liver injury or fibrosis manifested by the presence of many spots of focal cellular granulomatous lesions. Light microscopic observations revealed that DMN at 60 days caused massive hepatotoxicity compared to other treatments as control, yeast alone and yeast with DMN, including dissolution of hepatic cords, focal inflammation and necrotic tissues. Interestingly, 35 days of DMN alone also exhibited abnormal changes, including periportal fibrosis, degeneration of hepatic cords and increased apoptosis. Liver toxicity or injury was evident as disruption of tissue architecture.

Conclusion

DMN is able to induce liver injury in rats. Yeast has no side effect on the liver and on GOT, GPT and ALP, but induced expression of both IL-1α and IL-7r. The longer the yeast feeding time, the better the GOT, GPT and ALP activities, and the liver histopathology. IL-1α and 1L-7r have different responses for each of DMN and the yeast which proves that they are very sensitive for any abnormal compounds inside the rats' bodies. This study shows the importance of using yeasts as probiotics in the protection against liver fibrosis.

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