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Research Article

EFFECT OF 40% DIETARY RESTRICTION ON PERIODIC ACID SCHIFF (PAS) POSITIVE MATERIAL AND CYTOPLASMIC LIPID STAINING IN THE MOUSE HEPATOCYTES

K.H. Pathan[1], R.B. Yadav[1], K.A. Gajare[2] and A.A. Deshmukh[1*]
[1]Cellular stress response laboratory, Cell Biology Division, Department of Zoology, Shivaji University, Kolhapur
[2]Department of Zoology, The New College, Kolhapur
**Author for Correspondence: ashish_cellbio@rediffmail.com*

ABSTRACT
Practice of dietary restriction is known to mankind since time immemorial. However, the scientific evidences of its beneficial effects were documented in twentieth century. Dietary restriction is known for extending the lifespan in wide range organism from invertebrates to vertebrates. It is also known for reducing the morbidity of chronic diseases like diabetes, cardiovascular diseases, neurodegenerative disorders and diseases associated with ageing. In the present investigations, the effect of 40% dietary restriction was studied on PAS positive material and cytoplasmic lipids in the mouse hepatocytes. The animals were divided into two groups as *ad libitum* fed group and 40% diet restricted group. The diet restriction was initiated after three months of age and continued up to eighteen months. The PAS positive material and cytoplasmic lipids in the hepatocytes were histochemically stained by PAS and Oil Red O staining technique respectively. In the *ad libitum* fed group, the PAS positive material was homogenously distributed in the hepatocytes, whereas, in the diet restricted group, it was predominant in the sinusoidal space and just beneath the plasma membrane indicating the release of glucose by glycogenolysis. There was lower staining intensity of cytoplasmic lipids in the hepatocytes of diet restricted group as compared to the control group suggesting the beneficial role of dietary restriction in reducing the cytoplasmic lipids in the hepatocytes.

Key words: Dietary restriction, PAS positive material, Oil red O, cytoplasmic lipids, hepatocytes.

INTRODUCTION
Dietary restriction is the reduction of daily food intake without malnutrition and without compromising the intake of essential nutrients (Canto and Zuwerx 2009). McCay (1935) reported for the first time that the dietary restriction is able to extend the lifespan. Dietary restriction is synonymously used with calorie restriction and food restriction. According to Trepanowski *et al.* (2011), calorie restriction reduces the morbidity of diabetes, kidney diseases, cardiovascular diseases, cancer, respiratory diseases, autoimmune diseases, neurodegenerative diseases etc. There are two paradigms in dietary restriction. The first is limited daily feeding in which 20-40% intake of diet is restricted than the *ad libitum* consumption (Anson *et al.*2003) and the other is alternate day fasting (Goodrick *et al.* 1983). According to Weindruch and Walford (1988), Masoro (2005), dietary restriction is the non-genetic, and non-pharmaceutical intervention in extending the lifespan in many organisms. According to Omodei and Fontana (2011), calorie restriction is the most effective nutritional intervention for slowing down the aging and preventing the chronic diseases in rodents.
Non-alcoholic fatty acid disease is recognized as a major health burden (Tetri and Caldwell 2003). It comprises of fatty infiltration of liver which is accompanied by inflammation (Matteoni *et al.* 1999). Although, the beneficial effects of dietary restriction are well known in terms of improving the insulin sensitivity, control of diabetes and delayed aging; the effect of dietary restriction on PAS positive material and cytoplasmic lipid content is not explored. Larson-Meyer *et al.* (2008) studied the effect of six month calorie restriction and exercise on serum and liver lipids. The Liver lipid content was assessed by magnetic resonance spectroscopy and computed tomography. The authors concluded that calorie

restriction may be beneficial for reducing liver lipid and lowering triglycerides in overweight subjects. In the present investigations, the effect of dietary restriction was studied on Periodic Acid Schiff (PAS) reactive material and cytoplasmic lipids in the Swiss albino mice. The 40% dietary restriction was commenced from the age of three months and continued up to 18 months.

MATERIALS AND METHODS

For the present investigations, male Swiss albino mice *Mus musculus* were used. All procedures were carried out with the due permission on Institutional Animal Ethics Committee and as per the guidelines of CPCSEA. Animals were reared in the departmental animal house under 12 hrs light and 12 hrs dark cycles. Animals were supplied with fresh water and pelleted feed daily.

Study groups: The animals were grouped into two groups : 1) *Ad libitum* fed group i.e. control group, which were supplied with plenty of feed and water and 2) experimental group i.e. 40% diet restricted group. In this group, the diet was 40% less than the *ad libitum* feeding. The dietary restriction was initiated from three months of age i.e. after attaining the sexual maturity and continued up to eighteen months. There were six animals in each group. The animals were weighed weekly to ensure that there was no malnutrition during dietary restriction.

Preparation of paraffin embedded blocks: After eighteen months, the animals were sacrificed by cervical dislocation. The liver was excised, minced into pieces, fixed in neutral buffered formalin for 24 hours, washed under running tap water for next twenty-four hours and serially dehydrated through ascending grades of ethanol Viz. 30%,50%,70%, 90% and finally into absolute ethanol, each wash was of 45 min duration. Thereafter, the liver pieces were cleared in xylene for 15 min and transferred into molten paraffin wax at 60^{o}C for 45 min for hot impregnation, which was followed by second wash of molten paraffin wax at 60^{o}C for another 45 min. The paraffin blocks were prepared and the transverse sections of thickness 6µ were cut on the rotary microtome. The histological structure of the liver was studied by routine haematoxylin-eosin staining technique.

Histochemical study of glycogen by PAS reaction (McManus 1948): The paraffin embedded sections of the liver were dewaxed in xylene for 30 min and hydrated through descending grades of ethanol Viz. absolute ethanol, 90%, 70%, 50%, 30% and finally brought to distilled water. The sections were treated with 0.5% Periodic acid for 5 min, rinsed with distilled water and stained with Schiff reagent for 30 min. The sections were treated with 0.5% sodium metabisulfite for 5min. rinsed with distilled water and dehydrated through ascending grades of ethanol, cleared in xylene and mounted in DPX.

Histochemical staining of cytoplasmic lipids by Oil Red O (Lillie 1976): The paraffin embedded sections of the liver were brought to distilled water as described above and treated with acetone for 2min. Thereafter, the sections were treated with Oil red O stain for 20 min at room temperature, which was followed by treatment of 50% isopropanol and rapid dehydration to absolute ethanol, clearing in xylene and mounting in DPX.

The slides were observed under microscope at 400X magnification.

RESULTS AND DISCUSSION

Results: Haematoxylin eosin staining of the hepatocytes of the *ad libitum* fed mice revealed intensely stained cytoplasm and hyperchromatic nuclei (Plate I Fig 1). In 40% diet restricted group, the cytoplasm and nuclei were normochromatic (Plate I Fig 2). In PAS technique, the PAS positive material was homogenously distributed in the hepatocytes of the control group (Plate I Fig 3). In 40% diet restricted group it was predominant in the sinusoidal space. The reaction was more intense beneath the plasma membrane at the periphery of the cytoplasm (Plate I fig 4). In Oil red O technique, accumulation of fat in hepatocytes was demonstrated. Oil red O was intensely deposited in the hepatocytes of the control group (Plate I Fig 5). The hepatocytes of 40% diet restricted group exhibited scanty staining with Oil red O (Plate I Fig 6).

Plate 1

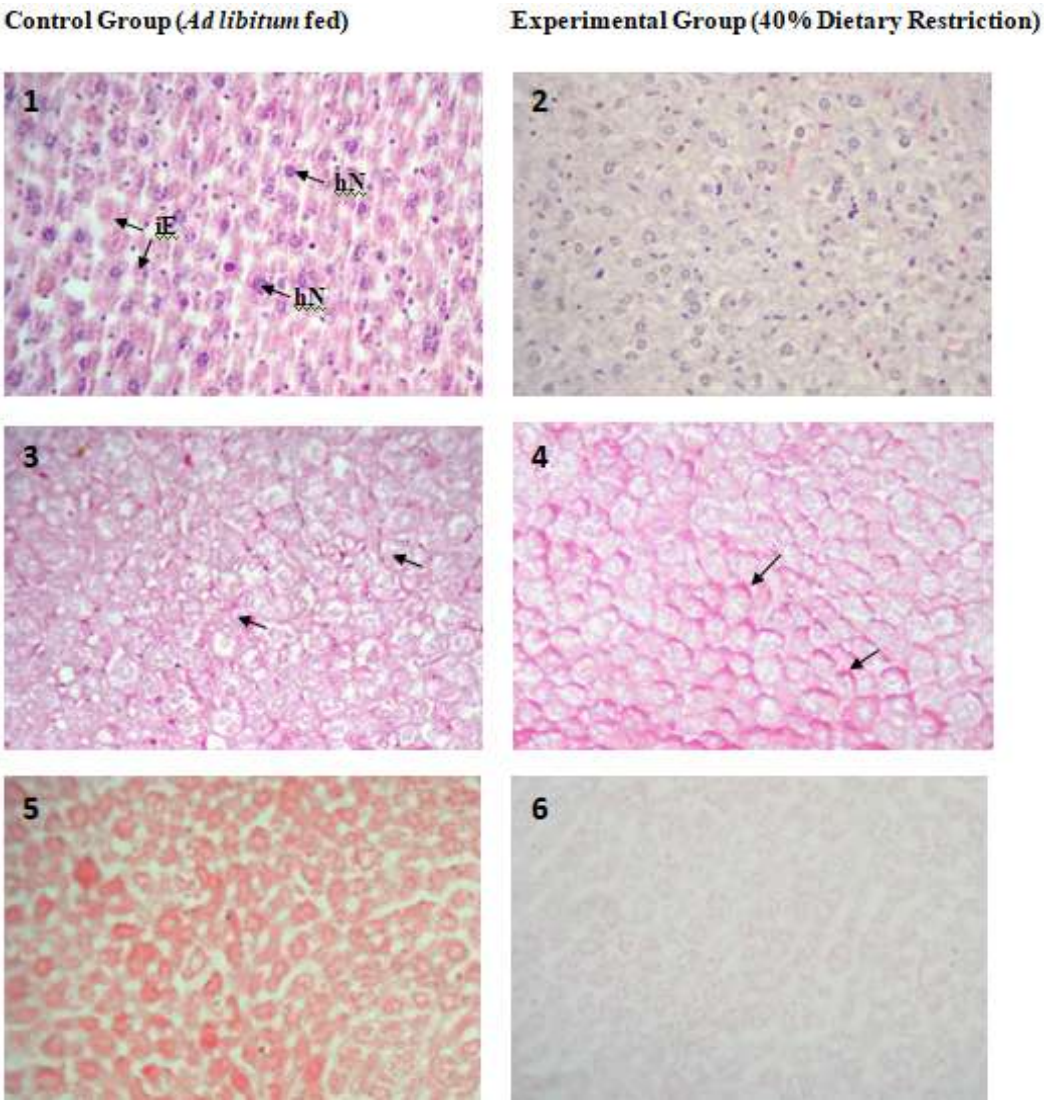

Fig 1 and 2 show hepatocytes stained with haematoxylin eosin technique. iE: increased eosinophilia, hN: hyperchromatic nucleus Fig 3 and 4 show hepatocytes stained with PAS technique. Arrows indicate PAS +ve material. Fig 5 and 6 show hepatocytes stained with Oil Red O showing orange staining. (400X)

Discussion: PAS staining technique is meant for histochemical demonstration of glycogen, glycoproteins and glycolipids (Thompson and Hunt 1966). In the hepatocytes of *ad libitum* fed mice there was homogenous distribution of PAS positive material. This indicates deposition of glycogen, glycoproteins and glycolipids in the hepatocytes. In the present investigations, the intensely stained PAS positive material just beneath the plasma membrane and in the sinusoidal space of the liver of diet restricted group indicates the site of glycogenolysis and release of glucose into the sinusoids (Rui 2011).

Oil red O stains triglycerides and lipoproteins (Horobin and Kiernan 2002). In the hepatocytes of *ad libitum* fed group, the intense staining with Oil red O indicates higher content of lipids. In the 40% diet restricted group, the scanty staining of hepatocytes with Oil red O suggests lower cytoplasmic lipid content. In *ad libitum* feeding, the excess of acetyl CoA is used as a precursor for synthesis of fatty acids

(Fell and Small 1986) and the free fatty acids are conjugated with glycerol moiety to form triglycerides which are stored in the liver and adipocytes (Kawano and Cohen 2013). In 40% diet restricted group, low intensity of Oil red O staining suggests mobilization of fats from the liver. Whenever there is inadequacy of the calories, the free fatty acids from the triglycerides are made available to generate energy by beta oxidation (Cahill 2006).

Accumulation of lipid in the hepatocytes called fatty liver is an abnormal form of liver structure and function, which progresses with age (Dam-Larsen *et al.* 2004, 2005). The increased Oil red O staining in the hepatocytes could be an age dependant accumulation of fat, since the animals were of 18 months of age. However, in diet restricted group, even though the animals were of the same age i.e. 18 months, there was scanty staining with Oil Red O suggesting no accumulation of fat in abnormally higher form indicating the beneficial effect of dietary restriction in preserving the cellular health of hepatocytes to youthful condition. Weindruch *et al.* (1986) demonstrated that the Dietary restriction retards the aging processes in mice. According to Masoro (1993), dietary restriction helps maintain many physiological processes in a youthful state and slows down almost all age associated disease processes. Mockett *et al.* (2006) reported that dietary restriction significantly increases the life span in mice. In the present investigations, dietary restriction was found to be beneficial in maintaining the health of the hepatocytes. However, further biochemical studies, glycogen specific histochemical studies are required to strengthen these findings.

Conclusion: 40% Dietary restriction helps to reduce the cytoplasmic lipids in the liver.

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