The influence of cyclic feeding regime on the RNA/DNA ratio of the hepatocytes nuclei of young and old rats

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***Abstract*- RNA mediates the translation of instructions from DNA into protein. Experimental studies by McCay et al 1935 showed that CR has beneficial effects in longevity, age related diseases, cancer and adaption to stress in rodents.**

In this study we investigated the effect of CFR a method with CR effects, on the RNA/DNA ratio in the hepatocytes nuclei of 3 months old rats and 20 months old rats. Fasting may enhance health by destroying certain unwanted transcripts and also increasing some that are important, especially those of enzymes involved in some metabolic pathways (Dhahbi et al., 1999)

The 3 months old rats results showed an immediate drop to 49% in the RNA/DNA ratio after the first fasting regime as compared to the controls. A further decline to 25% was observed after the first feeding, the second fasting showed an increase to 58% then a subsequent drop to 20% after the second re-feeding. While in 20 months rats a 68% decrease in RNA/DNA ratio was observed after the first fasting period, The fasting- feeding cycle initiated a slight rise to 80% in the DNA/RNA ratio, with a 70% and 77% RNA/DNA ratio following the second fasting and re-feeding cycle.

***Index Terms*—RNA/DNA ratio, Calorie restriction, chromatin**

I. Introduction

The RNA is a molecule that mediates the translation of the instructions from DNA into protein.(Friedman et al., 1996).Biochemical indicators, such as RNA concentration or the RNA:DNA ratio, are routinely used for estimating growth rates and nutritional condition (Buckley, 1984). Because RNA is a crucial component of protein synthesis, its concentration in tissue often mirror the rate of protein synthesis. The RNA:DNA ratio provides an index of the protein synthetic capacity per cell since the amount of DNA per cell is assumed not to vary with condition or with growth rate (Bulow, 1987).Furthermore the RNA/DNA ratio in quiescent cells, which are arrested in G0 phase of the cell cycle have lower level of RNA compared to active G1 phase. Animal development from a single-cell zygote to a fertile adult requires many rounds of cell division. Dependent on the environmental and developmental signals, cells in G 1 may temporarily or permanently leave the cell cycle and enter a quiescent or arrested phase known as G 0.(Albertson et al., 1978)

Several studies have demonstrated that in the Fischer 344 rats model, aging is associated with increase proliferative activity in numerous tissues notably of the gastrointestinal tract(Atillasoy and Holt, 1993; Holt and Yeh, 1988a, 1988b; Johnson and McCormack, 1994; Majumdar and Arlow , 1989; Majumdar et al 1988a,1988b) and a boost in transition from G1 to S phase (Xiao et al., 1999) which can be associated with the increase in the rate of transformational activities of proto-oncogenes to oncogenes (Boundless."Proto-oncogenes" Boundless biology .2017, Barker et al., 2001, Porter et al., 2010,Mikheev et al., 2009)

Gene expression regulation is key in the control of insulin expression so it gives a signal for blood glucose regulation, X-Chromosome inactivation in female mammals to prevent an overdose of the genes it contains and cyclin expression levels which governs progression through the eukaryotic cell cycle.

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Generally, gene expression is regulated through changes in the number and type of interactions between molecules that together influence the transcription of DNA and translation of RNA.(Zaidi et al., 2004; Mattick et al., 2009; Martinez and Walhout, 2009; Tomilin, 2008)In progression to cancer hundreds of genes are silenced or activated. Although some of these occurs by mutation (Vogelstein, 2013). Recent molecular studies of the aging process have focused on the oxidative modulations of cellular signaling systems and an alteration in gene regulation and expression (Matroule and Piette, 2000) Most gene regulation and expression activities are well known to be controlled by oxidatively sensitive transcription factors that undergo age related changes (Teillet et al., 2002; Kim et al., 2002) Calorie restriction exerts diverse benefits by its ability to counteract age related oxidative stress through the hormesis effects of gradual stress induction(Yu, 1996; Yu, 2000; Sohal and Weindruch, 1996; Yu, 2001a, 2001b)Base on these it can be proposed that the mechanisms which effectively suppress ROS are responsible for calorie restrictions anti-aging and anti-tumor action.

They experimental proofs that restriction in dietary intake (DR) has beneficial effects on longevity, age-related diseases, cancer and adaptation to stress in rodents were obtained in the early 1900s by Rous , and Osborne et al.In a hallmark study by "McCay et al (1935)" it was demonstrated that, compared with rats fed with a standard diet, rats fed under CR had longer life span, weighed less, showed heart hypertrophy, and had smaller livers, which they explained at the time as an effect of growth retardation. The free radical theory of aging states that every organism ages because of the accumulation of free radical damage which is mainly the result of reactive oxygen species production during cellular respiration in the mitochondria. Hence, the restriction in calorie consumption should reduce ROS and, as a consequence, delay aging and retard age-associated diseases. CR is difficult to carry out and sustain because of its applicability only after weaning and high mortality therefore an equally beneficial and easier approach which is CFR has been proposed by accumulating evidences (Sonneborn JS, 2005; Varady KA and Hellerstein MK, 2007). The purpose of our study was to investigate the influence of CFR on the RNA/DNA ratio in the hepatocytes nuclei of 3 months old rats and 20 months old rats. The Wistar rats were subjected to intermittent dietary restriction where the fasting days alternate the days with the provision of a limited amount of food afterwards the hepatocytes cell nucleus was isolated and the analysis of the RNA/DNA ratio was carried out using pyronin y which has binding affinity for both DNA and RNA and hoerst 33342 which blocks the binding of pyronin to the DNA and hence allowing the measurement of pyronin fluorescence spectra.

II. Materials and Methods

2.1. Animals and Treatment

The male rodents were obtained from Wistar rat colony and maintained at the Research institute of Biology, Vivarium under light and temperature controlled conditions (12-h light/dark cycle (7:00-19:00), 22°C ± 2°C), 50% ± 10% relative humidity and free access to water.

The animals were fed ad libitum with a pelleted feed developed in our laboratory (modified NIH-07 formula), its composition as follows: 33.5% wheat, 28% barley, 11% corn, 7% dried milk, 5.5% sunflower seeds, 5.5% dried fish, 5% dried brewer's yeast, 2% alfalfa meal, 1% chalk, 0.5% egg powder, 0.5% dietary salt mix, 0.5% gelatin. This dietary regimen was continued until 3 and 20 months of age for young and old groups, respectively.

At the time of the experiment, both young and old rats were individually housed and randomly assigned to one of five groups: (i) fed ad libitum (control); (ii)(F1) provided access to a limited amount of food every 48 hours, 4 g/100 g and 2 g/100 g of food/body weight for young and old rats, respectively for 10days(iii) (R1) fed with standard ad libitum for 20 days after F1 (iv)(F2) provided the same regimen as for F1 (v)(R2) Refed ad libitum .Feeding and cage cleaning were performed daily at the same time.

2.2. Nuclei isolation

Nuclei from rat liver were isolated according to the procedure described by Graham (Graham, 2004). Buffer A: Sucrose (25 mM), KCl (25 mM), MgCl2(5 mM), Hepes-NaOH (10 mM), PMSF (1 mM), pH=7.4. Buffer B: Sucrose (2.2 mM,), KCl (25 mM), MgCl2(10 mM), Hepes-NaOH (10 mM), PMSF (1 mM), pH=7.4. All solutions and tubes were kept in ice and all operations were carried out at 0–4°C. After overnight starvation, the experimental animals were sacrificed by cervical dislocation. Animal experiments was supervised and carried out by an authorized animal technician. The abdominal cavity was quickly opened and the liver was perfused with physiological solution and removed to a beaker containing about 30 mL of buffer A. The liver was transported to the chilled handy press (to remove blood vessels and the majority of connective tissue) and extracted to the homogenizer containing 25 ml of buffer B. The samples were homogenized by 7-8 tractions at 500-700 rpm of the pestle of Potter-Elvenjem homogenizer. The verification of homogenization effectiveness was performed by the phase-contrast microscopy. The homogenate was transferred into a 30-ml centrifuge tube (with the filtration through a single layer of nylon gauze (pore size 75µm) to remove the rest of the connective tissue) and centrifuged at 25,000g for 30min using refrigerated high-speed centrifuge with angle rotor. All the supernatant was decanted and the pellet was suspended again using a smooth Teflon pestle spinning at approximately 900 RPM in 25 ml of buffer B and centrifuged at 25,000 g for 30min. After decantation of the supernatant the nuclei-enriched pellet was separated from the nuclear debris by washing two times in buffer A at 1,500 g centrifugation for 10min. The purification quality of nuclei was checked by phase-contrast microscopy. The obtained nuclei were diluted with buffer A supplemented with 10 % (v/v) glycerol and frozen in liquid nitrogen until use.

2.3. RNA ASSAY

RNA content was determined using the method of "Darzynkiewicz et al (Darzynkiewicz, 1987)" adopted for fluorescence spectroscopy. The general feature of the method is the blocking of binding sides of Pyronine Y on DNA with Hoechst 33342. Pyronin Y was obtained from Fluka (Hauppauge, NY) (Cremer, 1985). (Pure pyronin Y is now available from Polysciences, Inc., Warrington, PA).

III. Results

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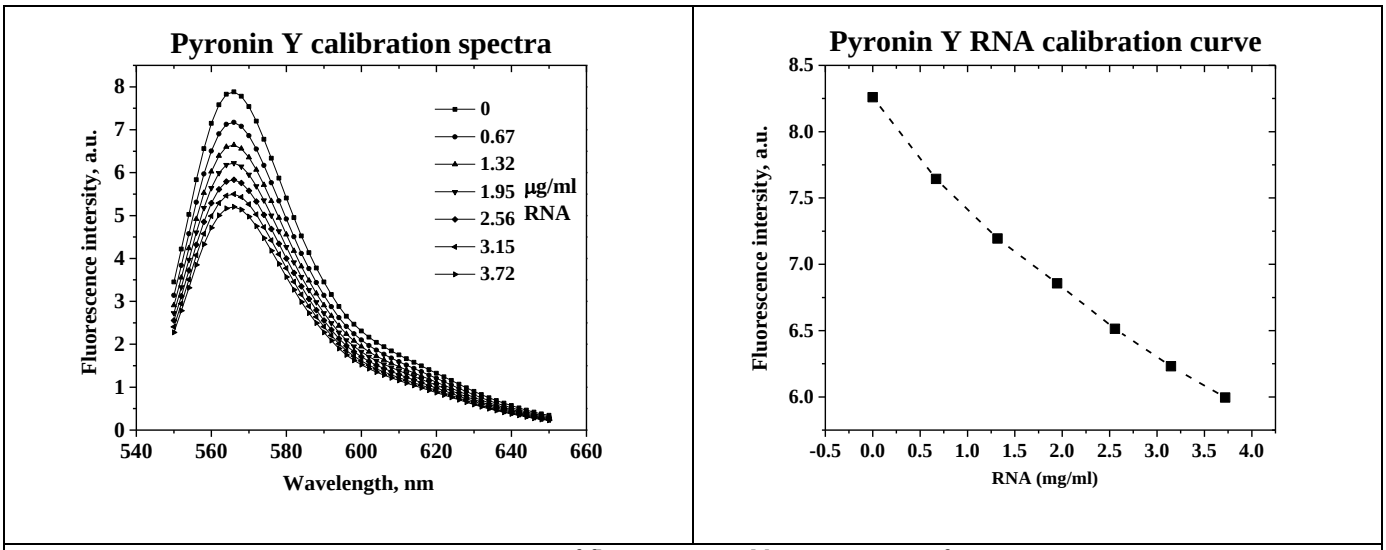

Fig.1. Demonstration of flourescence calibration spectra of RNA content

The DNA was blocked by hoesrt 33342 to enable the measurement of RNA using Pyronin Y in the absence of hoerst 33342 pyronin y will bind with both DNA and RNA making it impossible to differentiate. Hence the RNA content was determined by measuring the intensities of pyronin Y from the fluorescence intensities spectra, the fluorescence decreases with the concentration of the RNA as shown above. The concentration of the dye was measured calorimetrically at 507 nm using the molar extinction coefficient E = 3.78 x l04 M-l cm-l. To investigate the specificity of staining with PY following fixation, cells were suspended in Hanks' salt solution and incubated either with DNase I (Worthington, Freehold, NJ; 0.5 mg ml, 37"C, 60 min) or RNase A (Worthington, RASE, lo3 units per ml, 37"C, 60 min) prior to staining with PY. The treatment of cells with RNAse to test the specificity is based on the reduction of the intensities of pyronin y spectra after treatment. The presence of Hoechst 33342 in the staining solution increases the specificity of RNA detection by PY. For instance, at 6.6 µM PY and in the absence of Hoechst 33342, only 64% of the cell fluorescence is sensitive to RNase, However this percentage rises to 92% in the presence of the DNA fluorochrome. Thus Hoechst 33342 (Banerjee et al., 2006; Ehrlich, 1982) suppresses the stainability of DNA with PY as detected by fluorescence. Hoechst intercalates into the minor groove of DNA where its fluorescence has the highest rate at AT-rich regions .Hoechst 33342 is an exclusive DNA dye while pyronin Y reacts with both DNA and RNA ,In the presence of Hoechst ,Pyronin Y reaction with DNA is blocked and pyronin y stains RNA only. This is based on the principle that when cells are first stained with Hoechst 33342 and then with pyronin y it is possible to distinguish DNA from RNA. Hoechst 33342 and pyronin Y staining method are used for the separation of G0 and G1 cell cycle phases. RNA ratio assay using this technique also gives the transcriptional activities of the cell.

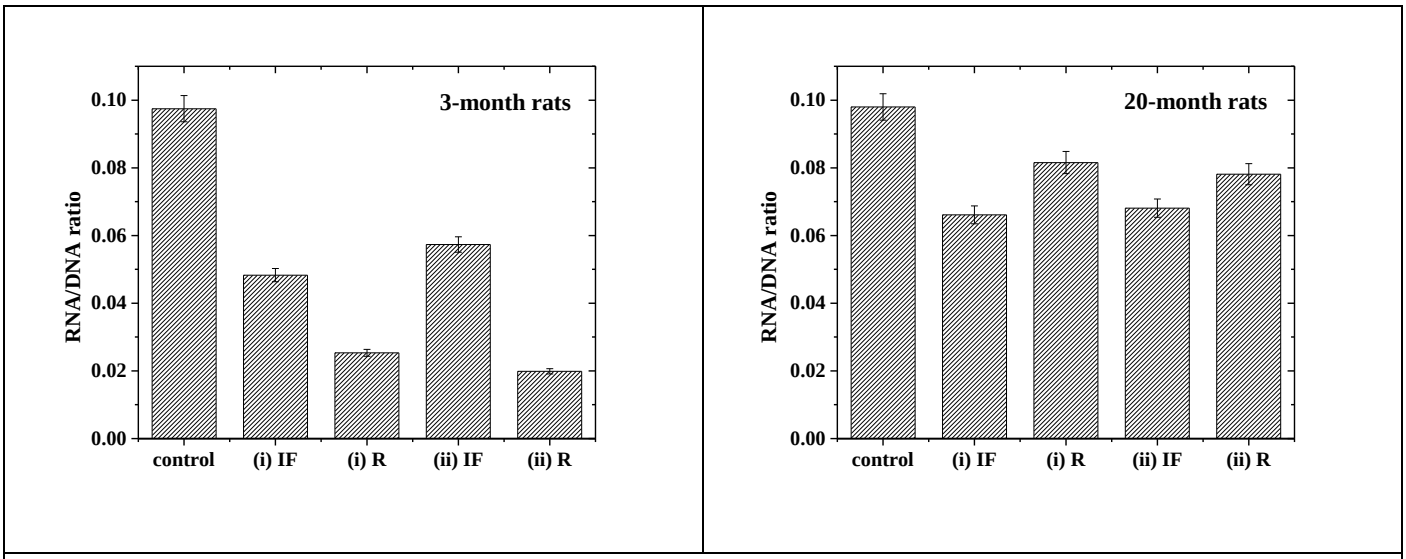

Fig.2. The RNA/DNA ratio of 3 and 20 months rats obtained from different representative fluorescence spectra not shown here using pyroninY which binds to RNA and hoerchst 33342 which specifically binds to DNA thus blocking the binding of pyronin y to DNA in the hepatocytes nuclei of young and old rats during the period of two cycles of intermittent fasting and re-feeding. Controls were fed with standard ad libithum. The first intermittent fasting went for 10days afterwards rats were re-fed

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with ad libithum for 20days the cycle was repeated. 10 rats used for each group.Intermittent fasting may play a role in health enhancement and provide anti-aging effects by reducing unwanted RNA transcripts.

The first fasting regime as envisaged lead to an acute drop in the RNA/DNA ratio to 49% and consequently a reduced protein synthesis as compared with the controls in the hepatocytes nuclei of 3 months old rats. A further decline to 25% after the first feeding was noted that may be by reason of certain epigenetic and post-transcriptional modifications that might have taken place during the first fasting period and were still in effect even during the first feeding and may be culpable for the low RNA/DNA ratio. The process of rehabilitation from these epigenetic changes and post-translational effects might have taken place gradually thus coinciding with the second fasting regime hence resulting in a rise to 58% of the RNA/DNA ratio which is a higher transcriptional active state coupled with an upsurge in protein synthesis which can be said to be as a result of the cumulating of the residual effect of the second feeding coinciding with the incoming second fasting regime. The understanding of the environmental regulation of DNA methylation in adult life is on the increase and current studies have shown that ageing and dietary factors can alter site-specific DNA methylation in human across different tissues.(Hannum G et al., 2013; Bacos K et al., 2016; Ronn T et al., 2015; Nitert MD et al., 2012; Brons C et al., 2010; Ghoshal K et al., 2006; Jacobson SC et al., 2014; Gillberg L et al., 2016)

Epigenetic modification have further been linked with differential gene expression and change in the metabolism in vital diabetic tissues, with that of the adipose tissue (Nillson E et al.,2014a, 2015b).Furthermore a study suggests that the promoter DNA methylation of two importantly adipokine genes, LEP and ADIPOQ, are affected by 36 hours fasting in SAT(subcutaneous adipose tissue) in NBW(normal birth weight)(Line Hjort et al., 2017). The second re-feeding led to another drop in the ratio of RNA/DNA to 20% which can be justified to be as result of post-transcriptional/epigenetic modifications that were about to take place due to the impact of the second fasting regime which then also coincided with the second re-feeding period, in light of these results one can speculate that the post-transcriptional modifications were quickly initiated in young animals but were slow to be reversed and that can be explained to be because of the chromatin structure usually observed in the tissues of younger animals which are more compact with higher integrity with less oxidative stress(ROS) impact thus adaptation takes a while to be achieved. The 20 months rats also showed a significant decrease to 68% in the RNA/DNA ratio in there hepatocytes nuclei after the first fasting period while the first feeding led to a slight rise in DNA/RNA ratio to 80% the same pattern was observed in the second fasting and second re-feeding regimes with 70% and 77% RNA/DNA ratio respectively, Demonstrating a steady suppression and activation of post-transcriptional/epigenetic activities, our results as interpreted showed that the old animals responded to the effect of the CFR in a consistent but limited manner thus indicating a more regulated response than in young animals and a chromatin structure with a more consistent adaptational activity, as a result of the epigenetic memory of previous events. They hepatocytes nuclei underwent epigenetic modifications slightly due to adaptive features so they transcriptional modifications that took place were easily reversed when the fasting regime was halted by feeding, Hence the suppressive effect of the fasting period was slight.

IV. Discussion

These results did not only provide an insight into the structural state of the chromatin by the measurement of the RNA content of the hepatocytes nuclei and the adaptation to the oxidative stress(ROS) of the fasting but also that RNA/DNA ratio can be used as a marker for understanding cell cycle arrest in higher organisms because most information on quiescence has been obtained from lower organisms. CFR exerts its influence on;

4.1. Metabolic genes regulation:

In the metabolic regulation of genes a further understanding into the mechanisms which control the processes of cell regulation during CFR will enable us to discern and develop suitable and safer strategies that will be effective in combating certain diseases that are associated with aging such as heart disease, diabetes and cancer, that result by way of poor gene expression control on the large number of metabolic and signaling pathways including sirtuins, the AMP-dependent kinase, the endocrine insulin/IGF-1, the nutrient-responsive kinases target of rapamycin (TOR), cyclic AMP-dependent kinase (PKA), Sch9/AKT (Zhu Y et al., 2013, Hardie DG,. 2004, Straus DS and Takemoto, 1990, Sahar E et al., 2014). The first fasting regime lead to a higher heterochromatin structure in young animals that already has a higher intergrity suggesting that as transcriptional activities increases with age, young animal cells are expected to have a low and more controlled transcriptional activities by virtue of the high proliferation in young animals cells. while in old animals there are more differentiated cells. Therefore a slight modification had a higher effect and the highly reduced RNA and transcriptional activity was observed in young animals while in old animals the heterochromatin is more loosely packed as a result of age linked increase in transcriptional activities because of a high population of differentiated cells.One could assume that any modification will only have a slight effect because of the aging associated increase wear and tear, transcriptional activity and expression of genes in various tissues which is probably to activate free radical scavengers that act as antioxidants, pro oxidants and those that are responsible for inflammation, which explains the slight and regular decrease and increase in RNA throughout the feeding regime in the old rats. This has shown the role of cyclic feeding regime in gene regulation and as a metabolic pathway regulator. A study found that CR reduces the expression of key enzymes of hepatic glycolysis and increases the expression of key enzymes responsible for gluconeogenesis and the disposal of nitrogen derived from muscle protein catabolism for energy production.(Dhahbi et al ,1999, Cao et al.,2001,Spindler,2001).

The studies also showed that CR reverses many of the age-related changes in the mRNA and/or activity of these key metabolic enzymes(Dhahbi and Spindler 2003). Fasting-refeeding kinetics studies in mice indicates that CR maintains

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higher rates of gluconeogenesis and protein catabolism even in the hours after feeding.(Lewis et al.,1985;el Hay et al.,1986;Merry et al.,1987;Goldspink et al.,1987;Merry and Holehan 1991;Dhahbi et al.,2001) CR rapidly induces fully reversible changes in gene expression, approximately, one-third of the CR-specific effects on gene expression are in genes related to energy metabolism and biosynthesis(Dhahbi et al.,2003)

Even after feeding CR still reduces glutamine sythetase activity and mRNA in the liver. CR decreases the expression of GH receptor in the liver of both young and old mice and induces overexpression of IGFI-binding protein-1 mRNA, Which inhibits IGFI signalling. The vital enzymes of gluconeogenesis PEPCK and G6Pase expression are induced by CR. (Dhahbi et al.,2003)

Several results suggest that CR reduces the enzymatic capacity of the liver for glycolysis(Dhahbi et al.,2001) According to studies the expression of acetyl-CoA acetyltransferase 1,fatty acid Coenzyme A ligase, Long chain 2,2,4-dienoyl-CoA reductase, liver fatty acid-binding protein-1,hepatic lipase and stearoyl-Coenzyme A desaturase 1 are reduced by CR.(Dhahbi et al.,2003)Merry and Holehan found that CR reduces the rate of protein synthesis 2.5 fold in young rats(Merry and Holehan,1991)

4.2.Oxidative stress regulation:

A number of investigators have proposed that CR may act by decreasing oxidative damage or enhancing its repair(Sohal and weindruch, 1996,Yu,1996).”ROS“ are generated as products of normal aerobic metabolism but their level increases under stress. Fasting inducing oxidative stress within the body as it leads to an upsurge in free radicals, the molecules mostly linked to aging. The free radical theory of aging describes aging as the accumulation of ROS that leads to cellular dysfunction overtime. The rise in free radicals is thought by scientist to be beneficial, by activating protective pathways ”Wegman said that if the body is intermittently exposed to levels of oxidative stress, it can build a better response to it”(Dawson C, 2016). Thus CR has been proposed as an etiology of oxidative stress described as nutritional stress thus to curb the deleterious effect of oxidative stress as the cell ages the gradual introduction of stress activities will lead to the development of innate form of defense mechanism by way of adaptation giving the cell an advantage when a greater stress condition that can lead to disease is encountered thus a future oxidative stress will not have any deleterious effect on the organism due to “hormesis”.

The survival of the cell during starvation is enabled by the production of ATP through the mitochondrial electron transport chain by oxidizing amino acids and fatty acids produced by autolysis of cellular material during autophagy.(Levine B and Yuan J, 2005)oxidative stress has been shown to induce autophagy when there is starvation(Matsui Y et al., 2007) High levels of ROS including free radicals e.g superoxide, hydroxyl radicals and hydrogen peroxide cause cellular damage and cell death under stress.(Scher-Shouval R et al., 2007) ROS are known to lead to the early degradation of mRNA transcripts hence the low RNA ratio observed during the fasting may be as a result of mRNA degradation due to the release of ROS by CFR which is a form of post-transcriptional regulation. Also ROS produced as a result of CFR can bind to transcriptional factors and further influence their activities. Multiple genes in eukaryotic cells involved in signaling pathways are regulated by oxidants. This control involves changes in the level of transcription, mRNA stability and signal transduction. The induction of mitogenic reponse is an example of the beneficial effects of ROS at low or moderate concentration. The role of free radicals in the reduction of ribonucleotides has been reported (Z.Durackova, 2010).

Increase in life span is achieved by removing ROS during CR.(Shaista P and Archana C, 2015) The control of oxidative stress and free radical production in response to CR seems to be mediated by various nutrient sensing pathways.(Ames et al.,1995; Civitarese et al.,2007)

4.3. Tumour cells dormancy:

It is a well-known fact that during starvation animals cells undergo senescence or apoptosis as a survival strategy, that is remaining dormant or getting rid of cells so as to reduce the workload of maintaining those cells (Scherz-Shrouval et al.,2010, Mathew R et al.,2007, Levine B and Kroemer G., 2008). The above studies also provides an insight on growth retardation through the Cell cycle analysis which is important for cancer research, anticancer agent screening which can give the cell cycle arrest phase of anticancer drugs (Evan and Vousden, 2001).This growth retardation/cell cycle analysis by fluorescence spectroscopy is based on the measurement of the DNA/RNA ratio. Which will be able to distinguish the G0 from the G1 phase. (Clenmesen, 1988; Gridelli et al., 2014; Ou, 2011).Hoechst 33342 binds DNA without interaction with RNA, While pyronin Y is a stain specific to RNA(Shapiro, 1981).In G1 phase the expression of RNA starts more than in G0 phase while the DNA holds diploid. At low RNA more cells can be said to be in the Go phase and high RNA ratio implies that more cells are proliferating .

Low RNA/DNA ratio is an indication of quiescence and in quiescence state cells are not dividing but are arrested in the cell cycle in G0-G1.(Aguirre and Julio, 2007)Dormancy have been documented in cancer cells as a characteristic of majority of tumor cells therefore an understanding of dormancy in cells will shed more light on the mechanism cells use to enter quiescence state and CFR has been able to cause a similar response as marked by the different RNA/DNA ratio levels corresponding to the dual states of the cells. The communications of cells and their microenvironment determines whether the cells will undergo arrest. This suggest that CFR plays a part in influencing the cells microenviroment thus regulating there cellular activity.

It was proposed that tumor cells dormancy is initiated when the microenvironment or new environment during metastasis is not permissive, in a situation such as cellular stress or a lack of available growth factors (Wikman et al., 2008; Baig et al., 2015; Ranganathan et al., 2006)thus these cells can remain in this state for a long time and be clinically undetectable.(Aguirre and Julio, 2006)There are suggestions of a direct link between nutritional availability and the inhibition of protein synthesis. This mechanism protects tumor cells during acute nutrient deprivation, meaning that it is a key switch in the fate of these cells (Leprivier et al., 2013).

V. Conclusion

RNA ratio can be adopted as a marker for gene expression, quiescence and growth retardation. The understanding of the mechanism of cyclic feeding regime on RNA/DNA ratio and

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other related mechanisms would not only play a crucial role in enhancing healthy aging but also in the treatment of the diseased aging group especially those with cancer by potentially preventing the onset of cancer as well as allowing developed cancer cells to enter and stay in a weak or fully quiescence state because cells enter dormancy when they cannot adapt immediately to stress or a new microenvironment and also devising a strategy for the treatment of other age related diseases. Though quiescence has only been observed mostly in unicellular organisms but if the mechanism of cell cycle arrest can be understood and replicated in higher organisms it will be pivotal. The push for more understanding of the mechanism of quiescence is important for clinical reasons especially in cancer therapy because dormant cancer cells are often untreatable due to drug resistance. These cells are usually resistant to chemotherapy because they are not dividing and chemotherapy best targets rapidly dividing cells.(Aguirre and Julio, 2006; Naumov et al., 2003, Ranganathan et al., 2006)Thus a further understanding can allow the development of age specific strategies to combat other diseases such as diabetes, artherosclerosis, Huntington disease, That occur as a result of poor gene regulation and maybe CFR extends life by reducing the RNA concentration. Reduced RNA concentration can be proposed as one of the key mechanisms in anti-aging effect.

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