Amalakirasayana feeding declines the DNA Damage in wistar rat Cerebellum, Liver and Testis, through age

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Abstract
The genomic stability has been validated in tissues of the Wistar NIN rats in terms of Single Strand Breaks (SSB) and Double Strand Breaks (DSB), by oral feeding Amalakirasayana, supplied to us by experts of Aryavaidyasala, kottakkal, Kerala, India, for a period of 3 and 9 months starting at the animal age of 6 months. After successful feeding, the rats were sacrificed and checked for the analysis. Making use of the enzymes E. Coli Pol I (Pol I) and Calf Thymus Terminal Transferase (TdT) the SSBs and DSBs were measured. The results were significant in the experimental rat group, compare to the control. These results convincingly indicate that, the rasayana fed animals showed significantly less number of DNA damage than in the control animals, which points out the valuable impact of rasayana therapy towards the maintenance of stable DNA damage through age. We estimate that, the distinct decrease in the DNA strand breaks of rasayana fed animal results could be because of the increased repair capacity of the damaged DNA and the anti-oxidant action of the rasayana concoction with blended additives.

Key words: Ayurveda, Amalakirasayana, Aging, DNA Damage, tissues

Introduction
The DNA damages every day and it gets repaired constantly by an enzyme called polymerase β(1,2). But at some point of the age the enzyme capacity declines, this decrease in the repair capacity and the building up of the damaged DNA increases in the body (3) and lowers the overall metabolic activity which leads to death. Aging is a gradual change in an organism that lowers the immunity, muscle loss, physical and mental health (4) which increase the disease causing condition and other aspects of knowledge which involves gradual molecular modifications that leads to the biochemical differences with tissue imbalance (5).

Ayurveda is one of the world’s oldest holistic healing systems that developed thousands of years back in India, and now it is considered as an alternative medicine globally (6). It is believed that the health and wellness depend on a delicate balance between the mind, body, and spirit(7). Ayurvedic rasayanas are the powerful anti-oxidants and the natural healing systems which are well known to be responsible in restoring cellular equilibrium and maintain overall physiological balance(8,9).

As the scientific proof and the validations are lagging for ayurveda, it is been disregarded since decades, but now it has the surge of interest in the traditional medicine(10), of the affordable cost and the natural healing power of the rasayanas, now it is used in the practice. The concoction of the rasayana prepared by the pioneers of ayurveda from the plant extracts and
some of the herbs have a great effect in long run. One such ayurvedic rasayana, *Amalakirasayana* has been orally fed to 6 months old wistar rats of NIN for a period of 3 months and 9 months to check the DNA damage levels of Cerebellum, Liver and Testis organs of male rats with the control group. We got the significant results in *Amalakirasayana* fed animals as compare to the normal control group.

**Materials and Methods**

**Animals:** The rat experimental methods were affirmed by the ‘Ethical Committee on Animal Experiments’ at the National Institute of Nutrition, Hyderabad, India. 32 male wistar NIN (WNIN) rats were procured from the institute at the age of 6 months from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad. The animals were kept up as two groups; *Amalakirasayana* fed (male, n = 16) and Control (male, n = 16), maintained in polypropylene cages (two animals in each cage). The *Amalakirasayana* was fed by oral gavages to the experimental group animals at 4.5 gms per kg body wt. of the animal, along with normal given diet, and the control group animals were fed placebo (same amount of buffer used for making the rasayana suspension) with normal diet. The animals were maintained at temperature of 22± 2°C, relative humidity 55 ± 10% and on standard lighting conditions (12-h light/dark cycle). Both the experimental and control animals were sacrificed at 9 months and 15 months age of the animal (which implies 3 and 9 month fed) within the stipulated time intervals and all the organs were collected and labelled including brain and they were snap frozen and stored in -80°C for further experiments.

**Chemicals:** *Amalakirasayana* was prepared and supplied by Aryavaidyasala, Kottakkal, Kerala, India. In brief, preparation of *Amalakirasayana* takes place in four stages; in stage 1 dried goose berry (emblica officinalis) is pulverized by using tyco pulveriser. At stage 2, fresh gooseberry juice is prepared by using a juice extractor. At stage 3, the products obtained at stages 1 and 2 are blended and dried at 55°C under low pressure of 700 mm in a vacuum tray drier. The dry mass thus formed is then pulverized and stages 2 and 3 are repeated another 20 times (Total 21 times trituration). This procedure takes about 2-3 months to complete. At Stage 4, the thick paste of *Amalakirasayana* is prepared by blending the dry powder with ghee and honey, the procedure mentioned above is adapted from the classical procedures recommended in ancient scriptures (11).

Pol I and (TdT) were purchased from Fermentas, and Thermo Fisher Scientific respectively. Call Thymus DNA, PPO, POPOP, Unlabeled nucleotides all dNTPs were obtained from Sigma chemicals Co. (St. Louis, MO, USA). Get™ DNA template kit was purchased from G-Biosciences (St. Louis, MO, USA). 2.5cm glass fibre filter was purchased from Millipore. Radio labelled [α³²P]-dCTP (PLC-102) was purchased from JONAKI-BRIT (Hyderabad, India). All other chemicals used were of analytical grade. The radioactive counts were taken in Tricarb Liquid Scintillation Counter, PerkinElmer (Waltham, MA, USA).

**Single and Double Strand Breaks Estimation from the tissue isolated DNA:** According to the GET™ DNA Template Kit instructions the Genomic DNA from various tissues was extracted and the quantification and estimation of the DNA purity is done by using Nanodrop™2000 (Thermo Scientific, USA) at 260 and 280 nm. The single and double strand breaks assays was performed based on the ability of Pol I (As per the company instructions, 1 enzyme unit catalyzes the incorporation of 10 nmol of deoxyribonucleotides into a polynucleotide fraction in 30 min at 37°C) to add nucleotides at a free 3'-OH group present in one of the duplex DNA strands, which was described earlier(12) and if the conditions are controlled so as to have a known number of nucleotides added at each of the free 3’-OH group, using the other strand as template, then it can be easy to back calculate the number of SSBs in a given sample of DNA. Assuming, each diploid cell in rat has 6 pico gm of DNA, the number of SSBs in a cell can be
Amalakirasayana, declines the DNA Damage in rats. Similarly the property of TdT to add nucleotides at 3’-OH group present at any blunt end of DNA without any need for a template, is exploited to assess the number of DSB in a given DNA sample and in a cell.

These assays was performed as described in our earlier paper(11) to explain in brief, for estimation of single strand breaks, the volume of the reaction is 50 μl which contained 40mM Tris-HCl (pH 8.0), 1mM β-mercaptoethanol, 4mM ATP, 7.5mM MgCl₂, 100 μM of dATP, dTTP and dGTP and 25 μM of dCTP, 1μCi of [α³²P]-dCTP (3000 Ci/mmol), 1 unit of Pol I and 500 ng of genomic DNA. The reaction was carried out at 37°C for 20 min and stopped by keeping on ice and by addition of 1 ml of chilled TCA (10% ) containing 10mM tetrasodium pyrophosphate. CT DNA and 200 mg of BSA were added as carriers. The samples were incubated on ice for 5 min and then centrifuged for 15 seconds at 12,000 rpm at room temperature (RT). The acquired supernatant was discarded and pellet was dissolved in 400 ml of NaOH (0.2 N). When the whole pellet was dissolved in NaOH then TCA (10%) solution (1 ml) was added to the dissolved pellet and centrifuged for 5 min at 6,000 rpm at RT. The acquired supernatant was discarded and the pellet was thoroughly resuspended in 500ml of chilled TCA (5%). The whole solution along with the precipitate was transferred onto a 2.5 cm glass fiber filters and washed six times each with chilled TCA (5%) and ethanol (95%) under a vacuum suction unit. The washed fiber filters were allowed to dry for 20 min in oven at 40°C or keeping in hood, overnight. The dried filters were taken in toluene based scintillation fluid (5 grams PPO and 0.5 grams of POPOP) per litre having 0.1% triton-X-100 and the radioactivity was counted for Disintegrations per Minute (DPM) of the individual sample with a Packard Tri-Carb Liquid Scintillation Counter.

Double Stranded DNA Breaks Estimation from the tissue isolated DNA: The estimation of double strand breaks was the similar method as in SSBs with a little change in the initial stage. The total reaction volume of 50 μl contained 100 mM sodium cacodylate buffer (pH 7.0), DTT (0.2 mM), CoCl₂ (1mM), 1μCi of [α³²P]-dCTP and 1 U of TdT (As per the company instructions, 1 U of the enzyme catalyzes the incorporation of 1 nmol of deoxythymidylicate into a polynucleotide fraction in 60 min at 37°C) and 500 ng of genomic DNA. The reaction was carried out at 37°C for 30 min. The remaining procedure is the same as described for single strand breaks assay.

Statistical Analysis: Statistical analysis was performed by Graph pad Prism 5.0.3 software (Graph pad Software Inc., CA, USA). Data were expressed as mean ± standard error of mean (SEM) of three independent experiments. Significance of difference between the groups was analyzed by Student’s t-test and one way ANOVA with Tukey post hoc test. A p-value \( P<0.05 \) (*), and \( P<0.01 \) (**) was considered significant for all interpretations and conclusions.

Results and Discussion

The results shown in a bar graph representation and the values on the bar graphs represent the damaged values in arbitrary units which was analysed by the graph pad prism software. In Fig 1, the single strand breaks were more in control with the damaged value of 13.6 and with an experimental value of 7.8 which shows significant at the 3 months of Amalakirasayana fed, when the feeding extended up to 9 months then, the results were equal in both the groups. Which implies that, even after long term feeding of the rasayana to the animals may not have effect on the DNA damage in Cerebellum, but it was not harming to the animal. In fig 2, the Liver DNA damage was much lower in the case of 3 months fed animals and significantly decreased in 9 months fed animals when compare to the control group. In Fig 3, the highly proliferating testis tissue had a significant value in the 9 months fed animals but not in 3 months fed.

When it comes to the Double Strand Breaks which are well known to be lethal to the body, have shown good effect in controlling the damages significantly, in all the tissues at 15
Fig. 1. Assessment of DNA-Single Strand Breaks, extracted from rat Cerebellum of control and Amalakirasayana fed for 3 months and 9 months through a sensitive biochemical assay which were tagged with radioactive deoxy nucleotide. ** indicates the P<0.01. This indicates that there is a significant decrease in the damaged DNA of 3 month feeding animals.

Fig. 2. Assessment of DNA-Single Strand Breaks, extracted from rat Liver of control and Amalakirasayana fed for 3 months and 9 months shows a significant decrease in the 9 month Amalaki fed animal's DNA. It has also the effect in 3 months but not significant.

Fig. 3. Assessment of DNA-Single Strand Breaks, extracted from rat Testis of control and Amalakirasayana fed for 3 months and 9 months. The 9 month Amalaki fed animals has a significant decrease in the damage of DNA. And the *indicates the P<0.05.

Fig. 4. DNA Double Strand Breaks of the Cerebellum extracted from the control and Amalakirasayana fed animals for 3 months and 9 months showing significantly decreased in the 9 months Amalaki fed. The DNA Double Strand Breaks are the distressing damages which occur in the DNA through the age. And they are even susceptible to cause DNA mutation which lead to the death of the animal.
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months aged Amalakirasayana fed animals (check Fig 4, 5 & 6). But in the liver tissue, Fig 5 showed significance in 3 months and as well as in 9 months Amalakirasayana fed animals. The observed pattern of the results thus implies that, the rasayana therapy confers significant protection against the forthcoming ailments, especially through age.

Conclusions

The rasayanas are said to be more effective when they are taken in an empty stomach, which we did for the animals in our study. The results clearly explain that, upon feeding the Amalakirasayana, animals have showed less number of damages compare to the control in long term intake of the rasayana. The Amalakirasayana which was a concoction of gooseberry, honey and ghee, which are known to have abundant anti-oxidation properties and with the honey and ghee concoction taken in an empty stomach, would have enhanced the mechanism of controlling the DNA damage. We cannot estimate the root cause behind these encouraging results as of now but, the anti-oxidation property of the rasayana concoction and its ability to metabolise and maintain the healthy environment of the cell could be one of the reasons behind this rejuvenation.

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