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Systematic Review

In Vitro Evaluation of Apoptotic Effects of Different *Viruddha Samyogas* of *Kshira* in Human Hepatic WRL-68 cell line

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Abstract:
Background: *Viruddhahara,* the incompatible food combinations explained in *Ayurveda* has deleterious effects on the body even affecting the progeny. Detailed understanding of the cellular level impact of *viruddhahara* is imperative. In this study the apoptotic effects of incompatible combinations of *kshira* were evaluated.
Materials and methods: The apoptotic effect of *viruddha kshira samyogas* with *lavana, kadali, maasha, matsya* and *dadima* was studied on the human hepatic WRL-68 cell line using fluorescent assay with Acridine orange/Propidium Iodide dual staining method. The morphological changes were observed in all the groups.
Results: All the *viruddha* combinations of *kshira* exhibited apoptotic changes of varying degrees. Early apoptotic changes like nuclear fragmentation, chromatin condensation, blebbing of cell membrane and late apoptotic changes and secondary necrotic cells were the morphological changes observed in the groups. Cells treated with *kshira* alone showed limited late apoptosis changes while 90% of the cells were viable.
Discussion & Conclusion: *Viruddha kshira samyogas* have apoptotic effects on WRL-68 cell line. From this study, it is known that *viruddhahara sevana* is detrimental to health. Eating habits and lifestyle plays vital role in causation of diseases. Hence, choosing diet as suggested in *Ayurveda* texts is a promising target for lifestyle interventions.

Keywords: *Viruddhahara*, Apoptosis, Cell Morphology, *Samyoga Viruddha*.

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INTRODUCTION:

Apoptosis or programmed cell death is a highly regulated energy dependent mechanism which maintains tissue homeostasis. Several morphological and biochemical changes occur in apoptosis. Disruption in the balance of pro-apoptotic and anti-apoptotic proteins lead to dysregulated apoptosis. Aberrantly regulated apoptosis is associated with pathogenesis of many diseases including cardiometabolic diseases, inflammatory intestinal diseases and cancer[1]. Oxidative stress is found to be the underlying mechanism behind age induced apoptosis through free radical accumulation and mitochondrial DNA damage resulting in many degenerative diseases[2]. Free radical accumulation causes degradation of nucleic acids, proteins and cellular components. Pandey et al elaborated the role of cumulative free radical damage in pathogenesis of several diseases like allergies, arthritis, atherosclerosis, multiple sclerosis, parkinson's disease etc[3]. In addition to this, Pandey et al suggest free radical damage as the causative factor for all chronic degenerative diseases[3]. Dietary factors may act as

proapoptotic or anti-apoptotic which may cause insufficient apoptosis or excessive apoptosis leading to diseases.

Viruddhahara is explained as the *nidana* of many systemic diseases in our classics. *Viruddhahara* or incompatible food combinations and faulty food processing may release free radicals which in turn induces oxidative stress resulting in excessive apoptosis. Antagonistic effects of different food combinations and pro-apoptotic compounds in incompatible food combinations may contribute to degenerative diseases and auto immune diseases. Anti-apoptotic compounds or dietary compounds that interfere with intrinsic and extrinsic pathways of apoptosis may cause suppression of apoptosis resulting in abnormal cell proliferation and carcinogenesis[4].

Viruddhahara not only implies mixing together incompatible food items but also includes the time of food consumption, order of consumption of different food items, quantity of food items and several other factors. *Kshira* (Milk) with *lavana*, *kshira* with *kadali* is categorised under *rasa viruddha* and *samyoga viruddha* by different *Acharyas. Kshira* with *maasha* is *rasa viruddha* according to *Sangrahakaara. Kshira* is incompatible with *matsya* due to opposite *virya.* Intake of *Kshira* with *amla phala dravyas like dadima* comes under *rasa viruddha* and *samyoga viruddha.* Sabnis et al in their study opine that *viruddhahara* have molecular level impact in the body. From previous studies, it is known that incompatible food combinations disrupt tissue homeostasis by dysregulating apoptotic mechanism[5,6,7]. Hence in the present study, the apoptotic effect of combinations of *kshira* with *lavana, kadali, maasha*, *matsya* and *dadima* was studied.

Materials and methods:

a) **Preparation of Materials:** *Kshira, lavana, kadali, maasha*, *matsya* and *dadima purchased* from market were authentified based on API guidelines. Chemical analysis of all the food materials was done. Each combination of *kshira* is made by mixing equal quantities of *kshira* with equal quantities of *lavana, kadali, maasha, matsya* and *dadima* separately.

b) **Apoptosis Fluorescent Assay by Dual AO/PI Staining method:** The WRL-68 cell line (1×10^4) were added in 24-well plates and treated with different *viruddha samyogas* of *kshira, kshira* alone for 24 hours. The cells treated with *kshira* alone*, kshira* with *lavana, kshira* with *kadali, kshira* with *maasha, kshira* with *matsya* and *kshira* with *dadima* were represented as A, B, C, D, E, F, respectively. Cells treated with Dulbecco's modified eagle medium (DMEM), control group was described as G. The treated cells were taken and washed with phosphate buffer saline. 5 µL of the fluorescent dye stain acridine orange (AO, 100 µg/mL) and 5 µL of propidium iodide (PI, 100 µg/mL) was added to it. Fluorescent dual staining assay with AO/PI was used in the study to identify the early and late apoptotic and necrotic cells. With dual staining method, the dead nucleated cells will be stained red and live cells appear green. The morphological changes were observed by a Floid cell imaging fluorescence microscope (EVOS XL Core Imaging System, MA, USA).

Results:

All the groups B to F had significant changes in the cell morphology with necrotic and apoptotic cells (Figure 1). Considerable number of apoptotic and necrotic cells were observed in all the *Viruddha* combination treated groups compared to the group A. Group G showed limited necrotic and late apoptotic cells with more than 90% viable cells and hence found to be biocompatible. The cell morphology was unaltered in the group A and does not appear to be cytotoxic (Figure 2). Blebbing of cell membranes, nuclear margination, chromatin condensation, nuclear fragmentation, apoptotic bodies were the morphological changes observed. AO/PI staining revealed that after treating with *Kshira* and *lavana*, the cells showed typical morphological features of apoptosis including chromatin condensation, nuclear fragmentation, membrane blebbing, secondary necrosis. Thus, early and late apoptotic and necrotic changes were seen in Group B. In Group C, cells with nuclear fragmentation, membrane blebbing and necrotic cells were clearly observed. Group D exhibited nuclear margination, chromatin condensation and blebbing of cell membranes which were consequences of apoptotic trigger. Also, late apoptotic features like loose membrane and apoptotic bodies were observed. Nuclear margination was observed in group E with few late apoptotic features. In Group F, membrane blebbing, late apoptotic changes, secondary necrosis were observed. Groups B and C induced more apoptotic changes and necrosis compared to other *viruddha* combination groups.

Discussion:

Understanding the dynamics of food interactions is crucial for better metabolism. The bioavailability of the nutrients, absorption, and metabolism are dependent on the food-food interactions. The mixing of different food items may alter the intestinal flora. Thus, food-food interactions have direct impact in the health outcomes of the individual[8]. Food interactions can be categorised as complementary interactions, conflicting interactions, enhancing interactions, inhibitory interactions and synergistic interactions[9]. While complementary, enhancing and synergistic interactions have positive effect on the nutrient absorption and bioavailability, inhibitory interactions and conflicting interactions may affect the nutrient absorption negatively. Also, conflicting interactions may cause serious side effects in the long run.

Understanding apoptosis is important to gain in depth knowledge of several homeostatic mechanisms of body and trophological impact on such mechanisms. Several methods like morphological methods, biochemistry, immunology etc are employed for detecting apoptosis in the body. Wyllie et al reported that cell shrinkage, apoptotic body formation, agglutination of chromatin, DNA fragmentation can be noted in apoptotic cells[10]. The morphological changes in apoptosis events are decrease in water content in the cells thereby shrinkage of the cells, cytoplasm becoming dense, and disappearance of microvilli on cell surface. The apoptotic cells separate from normal cells in the early phase. Chromatin condensation, pyknosis, margination of chromatin, fragmentation of nuclei are early apoptotic changes observed. Degradation of cytoskeleton, nuclear debris and transformation of organelle compounds into apoptotic bodies occurs in later

stages but the contents of the cells are not released as the cell membrane is intact.

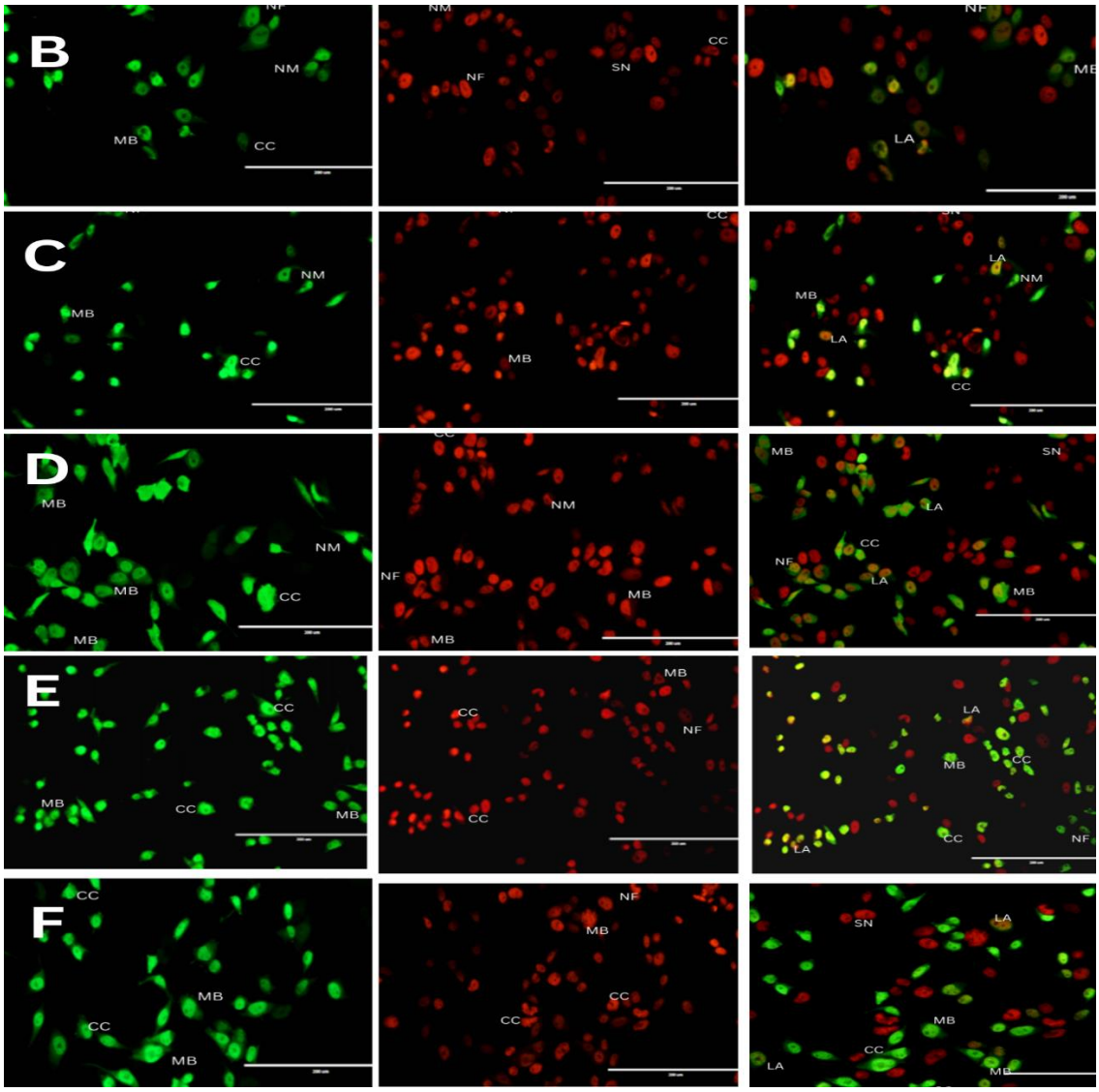

Figure 1: Fluorescent micrographs of AO/PI double-stained WRL-68 cell line. [B] WRL-68 cells treated with *kshira* and *lavana* showing nuclear fragmentation (NF), nuclear marginalisation (NM), chromatin condensation (CC), membrane blebbing (MB), late apoptotic events (LA), and secondary necrotic cells (SN). [C] Early apoptotic features like chromatin condensation and blebbing of membranes was observed. In addition, late apoptotic features were also observed in cells treated with *kshira* and *kadali* combination. [D] Nuclear marginalisation, chromatin condensation and membrane blebbing was obvious in cells treated with *kshira* and *maasha* [E]. Viable cells, chromatin condensation, blebbing of cell membrane, late apoptosis events were observed in WRL-68 cells treated with *kshira* and *matsya* combination. [F] Early apoptotic changes like cell membrane blebbing, chromatin condensation were observed in addition to late apoptotic features in *kshira* and *dadima* combination.

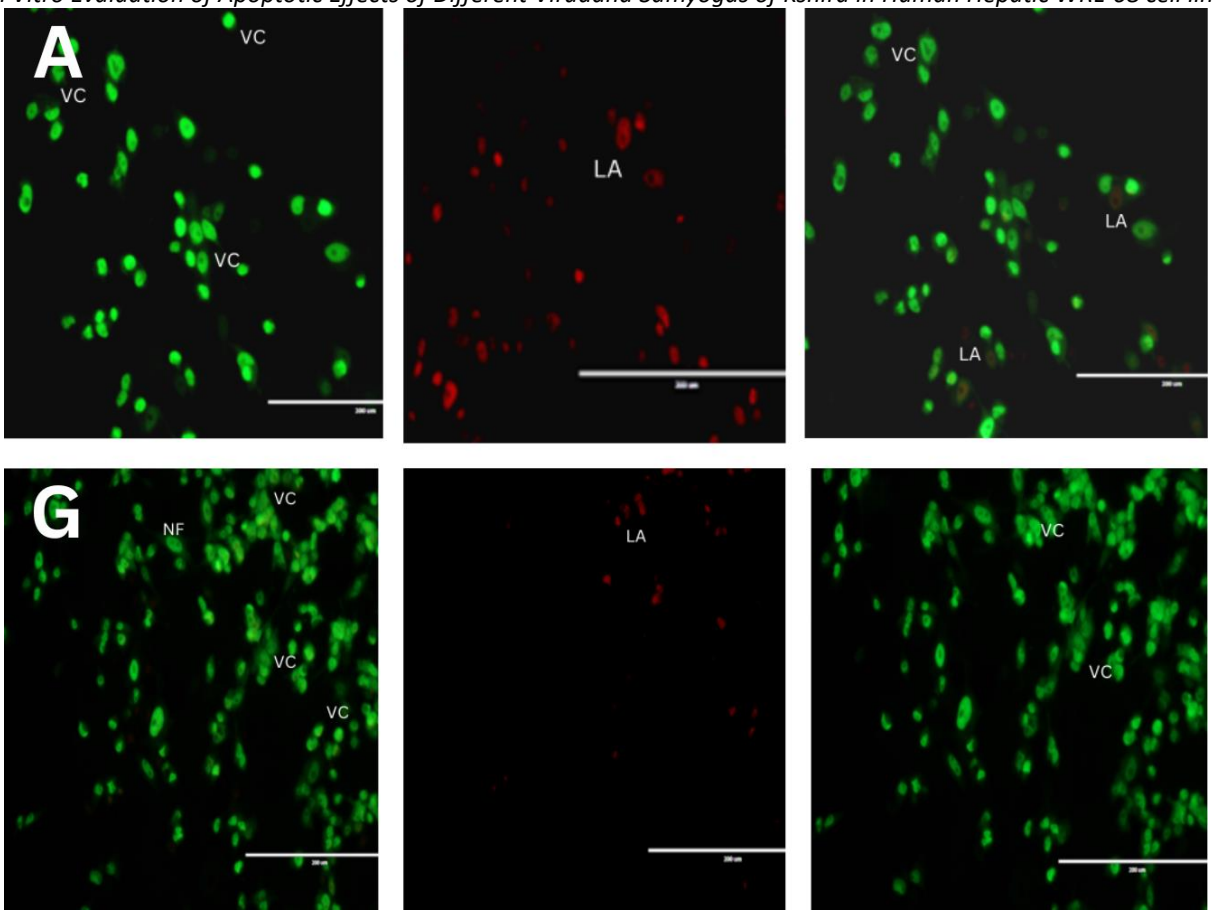

Figure 2: [A] WRL-68 cells treated with *kshira* alone reveals viable cells (VC) with few late apoptotic changes. [G] Control (untreated) WRL-68 cells showing normal cell morphology with no apoptotic cells.

Apoptosis detection based on morphological features can be done through microscopic examination. Fluorescent microscope or confocal microscopical examination after staining with Hoechst 33,342, dual acridine orange/ethidium bromide (AO/EB), dual Acridine Orange/Propidium Iodide staining, acridine orange (AO), or 4',6-diamidino-2-phenylindole (DAPI) staining reveals apoptotic changes in cells. In the present study, fluorescent microscopy was done after dual AO/PI staining. Acridine orange and Propidium iodide are flurochromes that are able to intercalate DNA and emit green and red fluorescence, respectively, revealing the live cells, apoptotic cells and dead cells. AO stain is membrane permeable and hence stains only live cells. These appear as cells with green nucleus. Chromatin condensation in early apoptotic cells will emit bright green signal in staining. Propidium stains late apoptotic cells and necrotic cells red. However, due to Forster resonance energy transfer, AO/PI double staining displays only dead nucleated cells fluorescence red and live nucleated cells in green[11].

The *viruddha* combinations were found to bring changes is cell morphology and apoptotic effect on the cells and thus does not exhibit biocompatibility and may have harmful effects on the body. In the present study, normal cell morphology without apoptosis was observed in the control (untreated) group. WRL-68 cells treated with *kshira* alone showed 90% of viable cells and only 10% were dead or apoptotic cells. Late apoptotic changes and dead cells were more in all the *viruddha* combinations of *kshira* when compared to cells treated with *kshira* alone. This proves that *viruddha* combinations are bio-incompatible and bring about increased apoptosis changes than that needed for homeostasis and disrupt the balance in the body. These increased apoptotic changes observed could be due to oxidative stress resulting from free radical damage.

Conclusion:

In the realm of nutrition, food-food interactions have far-ranging effects on nutrient absorption, glycaemic response and bioactive properties. *Ahara vidhi* is explained comprehensively in Ayurveda classics recognizing the interactions of different food substances. Concept of *viruddhahara* has undesirable consequences at cellular impact. Understanding and harnessing these interactions have a hand in improved dietary choices. This in turn will contribute to improved nutrient utilization, and overall health.

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