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Research Article

Alpha Stone Decoction, a Polyherbal Formulation, Induces Liver Mitochondrial-mediated Apoptosis in a Monosodium Glutamate Model of Hyperplasia

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Abstract
Studies have shown that reduced apoptosis is a recurrent theme of most tumorigenic cells. The mitochondrial Permeability Transition (mPT) pore is therefore an important target in the regulation of cell death because the release of apoptogenic proteins from the organelle causes cell suicide. Evidence of health hazards associated with MonoSodium Glutamate (MSG) is well documented. Our initial findings revealed that Alpha Stone Decoction (ASD), a polyherbal formulation used in the traditional treatment of uterine fibroids, reduced fibroblast cell count and deposits of collagen in the uteri of MSG-treated female Wistar rats. This study therefore investigated the effects of ASD on liver mitochondrial-mediated apoptosis in a model of MSG-induced hyperplasia. Twenty-four male Wistar rats were randomly divided into four groups with six animals in each group; A (Control), B (MSG only: 200mg/kgbw), C (MSG: 200mg/kgbw & ASD: 100 mg/kgbw) and D (ASD only: 100mg/kgbw). The administration was carried out as a single daily dose via intra-peritoneal route for 14 days, after which the animals were sacrificed. Mitochondrial swelling, mitochondrial ATPase activity, Caspase-9 and 3 activities were measured spectrophotometrically. Our data revealed that administration of ASD in a model of MSG-induced hyperplasia induced opening of the mPT pore in rat liver by 4.6 folds while the inductive effect was potentiated in animals administered both MSG and ASD by 5 folds. Similarly, ASD significantly enhanced ($p < 0.05$) mitochondrial ATPase activity, elevated levels of malondialdehyde and activated caspase-9 & 3 by 3.1, 2, 6 and 7 -folds, respectively, relative to levels observed in untreated animals. These effects were higher in animals co-administered ASD and MSG. Hence, Alpha Stone Decoction contains bioactive components that induce opening of the pore via generation of free radicals, depletion of ATP levels and activation of caspases 9 and 3. Therefore, ASD may mediate its anti-fibrotic effect in part via induction of mitochondrial-mediated apoptosis.

Key Words: *Monosodium glutamatate, mitochondrial permeability transition, alpha stone decoction, apoptosis*

INTRODUCTION

Currently, combination therapies are employed for the treatment of critical diseases in order to achieve enhanced therapeutic effects (Che *et al*., 2013). In the traditional system of Indian medicine, *Ayurvedic* herbals are prepared in a number of dosage forms in which most of them are poly herbal formulations (Srivastava *et al*., 2013). Their effectiveness, safety, low cost, availability and better acceptance have made poly herbal formulations an ideal treatment of choice especially in West Africa.

It is well established that apoptosis proceeds via any or both of two pathways; the extrinsic pathway which occurs when death ligands bind their cognate death receptors and the intrinsic pathway which requires that mitochondrial membrane permeabilization results in the release of cytochrome C into the cytosol (Halestrap *et al*.,1997; Locksley *et al*., 2001). The intrinsic pathway can be activated by a variety of receptor-independent stimuli such as radiation, free radicals, viral infections and serum/growth factor withdrawal (Indrian *et al*., 2001). This process is believed to occur when the mitochondrial Permeability Transition (mPT) pore opens (Kroemer *et al*., 2007, Letai, 2017) Indeed, the mPT pore is a voltage and Ca^{2+}-dependent, cyclosporine A-sensitive, high conductance channel, whose opening leads to a sudden increase in the permeability of the inner mitochondrial membrane to solutes with molecular mass up to 1500 Da (Bernadi *et al*., 2006) Prolonged mitochondrial permeability transition triggers molecular events such as matrix swelling, (Rasola and Bernadi, 2011) collapse of mitochondrial membrane potential, increase in inorganic phosphate concentration (Rasola and Bernadi, 2014) besides cytochrome C release (Karch and Molkentin, 2014**)** which eventually leads to cell death. Although the core components of the mPT pore is still a matter of debate, a number of chemicals have been shown to interact with the pore and modulate mitochondrial-mediated apoptosis (Millimouno *et al*., 2014, Oyebode *et al*.,

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2017). It is well established that tumours are characterized by low rate or total absence of apoptosis, (Fadeel *et al*., 1999a).

Uterine leiomyomas is a benign tumour of the uterus characterized by proliferation of smooth muscle cells (hyperplasia). It is known to be the most common category of solid pelvic tumors in females and has been tagged the disease of the African woman (Gerritsen, 2016). Although, the etiology of uterine fibroids is poorly understood, risk factors include genetics, hormonal predominance, race, diet among others. However, studies have shown that the black race has presented the strongest risk factor over the years (Stewart *et al*., 2017). Monosodium Glutamate (MSG) is a salt of glutamate improves taste and synthesized from L-glutamic acid and used as a flavour enhancer in foods. Although MSG enhances appetite, studies have shown that many health hazards are associated with MSG in humans and animals including its ability to promote liver inflammation and dysplasia in male wistar rats (Nakanish *et al*.,2008; Sharma *et al*.,2015; Mondal *et al*., 2017]. To this end, studies have proven that MSG can be used as models of obesity and uterine hyperplasia (Gobatto *et al*., 2002, DwiWahyun *et al*., 2014).

Alpha stone is a combination of acacia honey and different parts of plants such as leaves and acorns of Palestine oak (Quercus calliprinos) leaves and shoots of terebinth (*Pistacia palestine*), Golden chamomile, Carob (*Ceratoniasiliqua*), leaves of Dominican sage (*Salvia dominica*), leaves of field marigolds (*Calendula arvensic*),Tulips (*Tulipasystola*), shoots of the white broom (*Retama raetan*), the fruit of the bitter almond tree (*Amygdalius arabica*), seeds of fennel (*Foeniculum vulgare*) and shoots of the leafless ephedra (*Ephedra Foeminea*).

The ASD is known for its claims of high medicinal values including traditional management of fibroids. *Ceratonia siliqua,* commonly known as the carob tree has been reported to possess anti-inflammatory, anti-cancer and antiproliferative properties (Abdullatif, 2017**)**. *Ephedra* is one of the oldest known drugs (Dewick, 2002) isolated from *Ephedra foeminea* decoction and has *been* used widely by breast cancer patients to treat their ailments (Ben-Arye *et al*, 2016). Furthermore, it has been reported that Chamomile exposure induced apoptosis in cancer cells but not in normal cells at similar doses (Srivastava and Gupta, 2007).

Phytochemical screening of ASD revealed that the formulation contains flavonoids, phenols, saponins, steroids and tannins (Oyebode *et al*., 2019).Our initial findings showed that ASD significantly ($p < 0.05$) reduced fibroblast cell count in MSG-treated animals and also ameliorated the MSG-induced damage observed in the myometrium of the uteri and ovaries of the animals (Oyebode *et al*., 2019). We showed that increases ($p < 0.05$) in levels of total protein; triglycerides, progesterone, cholesterol and estrogen in the MSG-treated animals were ameliorated following administration of ASD. Having demonstrated the anti-fibrotic potential of ASD in our initial findings, it is not known whether mPT is implicated in this effect. Using a model of MSG–induced hyperplasia, we evaluated the involvement of liver mitochondrial-mediated apoptosis in the mechanism of action ASD.

Materials and Methods

Chemicals and Reagents

Monosodium glutamate, thiobarbituric acid, Cytochrome C (BDH Chemicals Ltd, Pools, England), 2,4-dinitrophenol, Folin-Ciocalteu's phenol reagent (2N) and all other reagents were purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA) and were of the highest purity grade.

Preparation of Alpha Stone Decoction

Alpha Stone Decoction was formulated from equal proportion of the aqueous extracts of the plants with enough quantity of Acacia honey. It is stable at room temperature. Alpha stone was obtained from a local distributor in Ibadan, Oyo State. It was pulverized to a fine powder using a mortar and pestle. The powdered alpha stone was stored at room temperature in a clean bottle.

Animal source, grouping and treatment

Male Wistar Strain rats (twenty-four) weighing between 100-120g were obtained from the Pre-Clinical Animal House, University of Ibadan, Nigeria. The rats were allowed to acclimatize for 14 days in the Animal House, Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria. They were kept in ventilated cages with 12 hours light/dark cycling and were given chow and water *ad libitum*. Doses of MSG (200mg/kgbw) and ASD (100mg/kgbw) were selected based on a previous study (Oyebode *et al*., 2019). The twenty-four male rats were randomly divided into 4 groups with six animals in each group; A (Control), B (200mg MSG /kgbw), C (200mgMSG /kgbw and ASD: 100mg/kgbw) and D (100mg ASD/kgbw). The administration was carried out by as a single daily dose via intra-peritoneal route for 14 days, after which they were sacrificed. The experiment was repeated twice.

Isolation of Rat Liver Mitochondria: Rat liver mitochondria were employed in this study because of its relatively high mitochondrial content. Mitochondria were isolated essentially according to the method of Johnson and Lardy (1967) and as modified by Olorunsogo *et al* (1979). The animals were sacrificed by cervical dislocation and the livers excised and trimmed to wash excess tissue. The livers were then weighed, washed with homogenizing buffer (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, pH 7.4 and 1mM EGTA), and homogenized as a 10%suspension in ice-cold buffer using a Porter Elvehjem glass homogenizer. The resulting homogenate was centrifuged in an MSE refrigerated centrifuge at 2300 rpm for 5 mins to remove the nuclear debris. This was done twice and the supernatant obtained was centrifuged at 13,000 rpm for 10mins to obtain the mitochondrial pellet. The supernatant was discarded while the pellet was washed twice with the washing buffer (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, pH 7.4, 0.5% BSA) at 12,000rpm for 10 mins. The mitochondria obtained were immediately resuspended in an appropriate volume of MSH buffer (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, pH 7.4) and immediately dispensed into Eppendorf tubes and kept on ice.

Mitochondrial Swelling Assay: Mitochondrial permeability transition was monitored by measuring changes in absorbance of mitochondrial suspension in the presence or absence of calcium (the triggering agent) in a T70 UV/visible spectrophotometer essentially according to the method of Lapidus and Sokolove (1992). Mitochondria (0.4mg protein/mL) were pre-incubated in the presence of 0.8µM rotenone in a medium containing 210mM mannitol, 70mM

sucrose and 5mM HEPES-KOH (pH 7.4) for 3mins at 27$_0$C prior to the addition of 120µM $CaCl_2$. Thirty seconds later, 5mM succinate was added and mitochondrial permeability transition quantified at 540nm for 12mins at 30secs interval. To determine the integrity of the mitochondrial membrane, 4mM spermine was immediately added following the addition of rotenone and just before the addition of mitochondria.

Determination of Mitochondrial Protein: Mitochondrial protein concentration was determined according to the method of Lowry *et al* (1951) using Bovine Serum Albumin as standard.

Assessment of Mitochondrial ATPase Activity: Mitochondrial ATPase activity was estimated by a modification of the method of Lardy and Wellman (1953) as modified by Olorunsogo and Malomo [(1985). Each reaction vessel contained 65mMTris-HCl (pH 7.4) 50 mM KCl, 1mM ATP and 250 mM sucrose in a final volume of 2mL. The reaction which was initiated by the addition of mitochondrial fraction (0.5mg protein/mL) was allowed to proceed for at 30$_0$C with constant shaking for 30 mins. The reaction was quenched by the addition of ice-cold 10% trichloroacetic acid. The precipitated protein was separated by centrifugation and the phosphate content of the supernatant was promptly estimated by a standard colorimetric method. The blank contained all components of the basic incubation medium including ATP but devoid of mitochondria while 2, 4-Dinitrophenol (2,4-DNP) was used as a standard uncoupling agent. The concentration of inorganic phosphate released following the hydrolysis of ATP was determined according to the method described by Olorunsogo and Bababunmi (1979). Briefly, 1.25% ammonium molybdate (1 mL) and 9% freshly prepared solution of ascorbic acid (1 mL) were added to 1 mL of the reaction mixture and allowed to stand for 30 min and absorbance was read at 680nm.

Estimation of Mitochondrial lipid peroxidation: Levels of malondialdehyde (MDA) were measured as index of lipid peroxidation in the test samples according to the method of Varshney and Kale (1990). An aliquot of 0.4 mL of test sample (mitochondria) was mixed with 1.6 mL of Tris-KCL buffer to which 0.5 mL of 30 % TCA was added. Thereafter, 0.5 mL of 0.75% TBA was added and the tubes were heated in a water bath for 45 min at 80$_0$C. The samples were then cooled and centrifuged at 3,000 rpm for 10 min. The clear supernatant was collected and absorbance was measured against a reference blank of distilled water at 532 nm. The MDA level was calculated using extinction co-efficient of 0.156/µM/cm (Adam – Vizi and Seregi [39]

Lipid Peroxidation (nmoleMDA/mg protein)

$$= \frac{\text{Absorbance x Volume of mixture}}{E_{532nm} \text{ x Volume of Sample x mg protein/mL}}$$

Determination of Caspase activities: Caspase-9 and 3 activities were assayed in liver post-mitochondrial fraction using a colorimetric substrate, Ac-LEHD-pNA and Ac-DEVD-pNA respectively in accordance with manufacturer's instruction from Biovision International (USA).Cleavage of the C-terminal peptide bond by the enzymereleased p-nitroaniline was measured at 405 nm. Mean values of triplicate measurements were presented and caspases 3 and 9 activities were calculated from the standard curve.

Statistical Analysis*:* Statistical analysis was performed with Graph pad (version 5.0) using one-way ANOVA. (non-parametric test) and Turkey's multiple comparison test was used. Level of significance was set at $p < 0.05$. All the results were expressed as mean ± standard deviation (SD).

RESULTS.

Effect of Alpha stone decoction (ASD) on mPT pore opening in rat liver mitochondria in a model of MSG-induced hyperplasia: Figure 1 shows the effects of alpha stone decoction on mPT pore in *vivo*. First, the integrity of the mitochondria from the untreated animal was ascertained prior to their use for further experiements. As seen from the figure, when mitochondria from control untreated animals were made to respire on succinate, a respiratory substrate, the representative profile obtained from change in absorbance at 540nm revealed that there was no significant change in the volume of mitochondria after a period of 12 mins. On addition of calcium, a standard triggering agent, large amplitude swelling of 10.7 folds was obtained which was almost totally reversed by spermine (83%), a standard inhibitor of pore opening. Thus, confirming that the mitochondria were intact before exposure to calcium and that they were suitable for use.

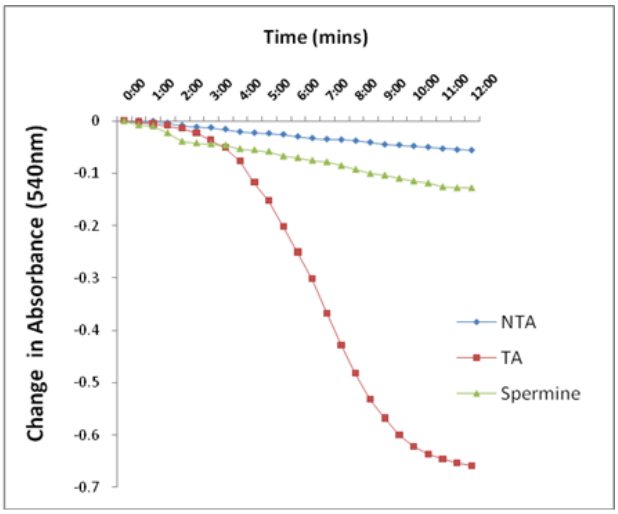

Figure 1:
Representative illustration of calcium-induced opening of normal rat liver mitochondrial membrane permeability transition pore and the reversal effect of spermine. NTA=No Triggering Agent, TA=Triggering Agent. Mitochondria were suspended in MSH buffer and energized by succinate and the absorbancemeasured at 540 nm. Spermine was added as inhibitor
*Typical data of one experiment are shown and similar results were obtained in at least three different preparations.

Furthermore, intraperitoneal administration of ASD (100 mg/kgbw) and MSG (200 mg/kgbw) separately to rats for 14 days induced mPT pore opening by 4.6 and 4.7 folds, respectively. Interestingly, this inductive effect was further potentiated in the animals that received both MSG and ASD (Figure 2).

Enhancement of rat liver Mitochondrial F_0F_1 ATPase activity by Alpha stone decoction (ASD) in MSG-induced hyperplasia

The effects of ASD on rat liver mitochondrial ATPase activity in MSG –induced hyperplasia in male Wistar rats are shown in figure 2. The rate of release of inorganic phosphate from the

ɤ- position of ATP was used as an index of enhancement of Mitochondrial ATPase activity. The results showed that mitochondrial ATPase was significantly enhanced ($p < 0.05$) at pH = 7.4 by separate administration of ASD (100mg/kg) and MSG (200mg/kg) by 3.1 and 3.2 folds, respectively when compared to mitochondria from untreated animals. Moreover, further enhancement ($p < 0.05$) of F_0F_1 ATPase activity was observed when animals were co-administered MSG and ASD (3.7 folds) in comparison with groups that received only MSG or ASD alone. Also, on addition of 2, 4-dinitrophenol, a standard uncoupler of oxidative phosphorylation, ATPase activity was enhanced by 5.3 folds

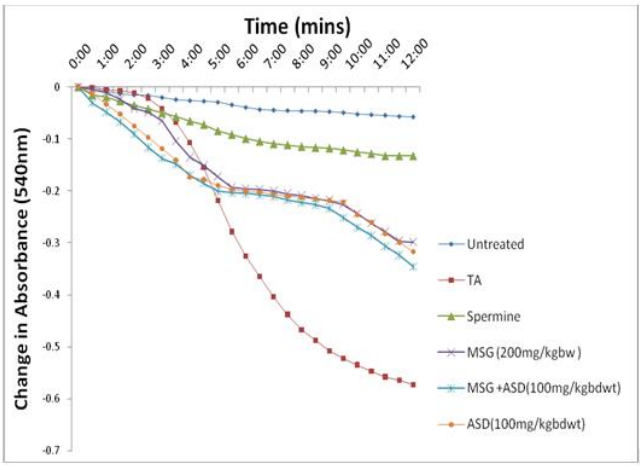

Figure 2:
Representative profiles of changes in absorbance of mitochondria when treated with Alpha stone decoction in MSG-induced hyperplasia model rats (14 days)
TA: Triggering agent. *Typical data of one experiment are shown and similar results were obtained in at least three different preparations.

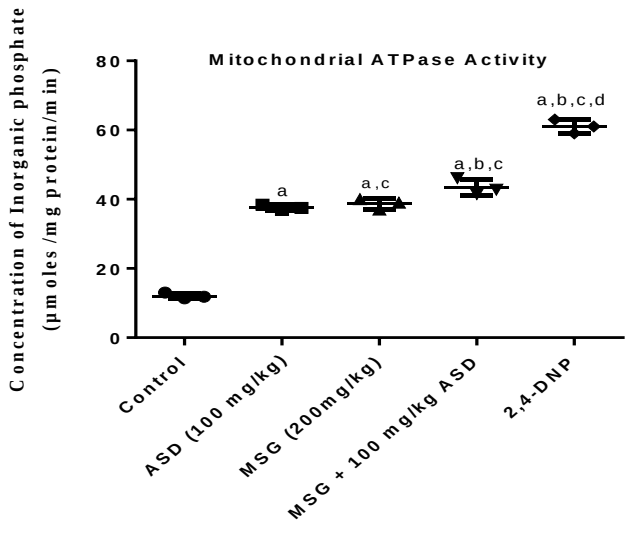

Figure 3:
Enhancement of Mitochondrial ATPase activity byASD in a rat model of MSG-induced hyperplasia.2,4-DNP- 2,4- dinitrophenol. Values are mean ± SD, $p < 0.05$ compared with control (a), $p < 0.05$ compared with MSG alone (b). $p < 0.05$ compared with ASD (c), $p < 0.05$ compared with MSG +ASD (d)

The *in vivo* effects of administration of Alpha stone decoction on mitochondria lipid peroxidation in a rat model of hyperplasia are depicted in figure 3. Levels of malondialdehyde generated were used as an indication of lipid peroxidation. The results revealed that the levels of malondialdehyde increased significantly ($p < 0.05$) in animals that separately received; ASD (100mg/kg), MSG (200mg/kg) and those administered both MSG and ASD when compared to untreated animals. In this regard, percentages of MDA were 50, 66 and 70 % in ASD (100mg/kg), MSG (200mg/kg) and MSG plus ASD co-administered groups, respectively. Furthermore, levels of malondialdehyde generated were significant ($p < 0.05$) between groups.

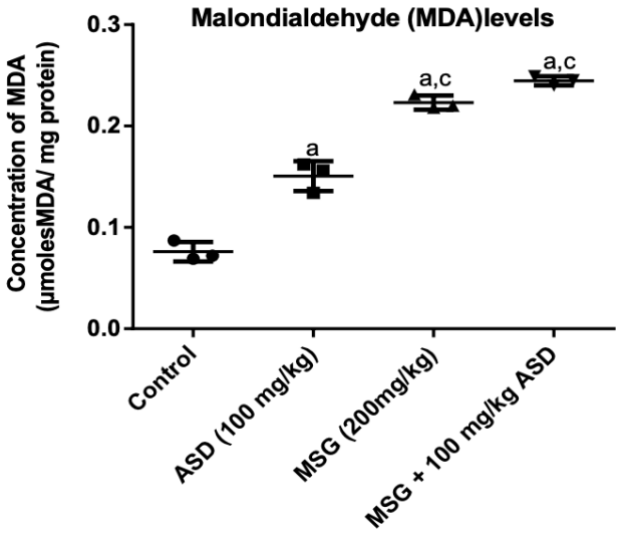

Figure 4:
Effects of alpha stone decoction on mitochondrial lipid peroxidation in a model of MSG –induced hyperplasia in male rats. Values are mean ± SD, $p < 0.05$ compared with control (a), $p < 0.05$ compared with ASD (c).

Alpha Stone Decoction activates caspase-9 and 3 in a rat model of MSG-induced hyperplasia: To establish if opening of the pore by ASD in the rat model of MSG-induced hyperplasia resulted in mitochondrial-dependent cell death, we assayed for the activation of caspases 3 and 9 in the post-mitochondrial fraction. The results obtained showed that initiator caspase-9 and executioner caspase-3 were significantly ($p<0.01$) elevated following administration of ASD in male Wistar rat -model of MSG-induced hyperplasia (figure 5). In this regard, a fold increase of 6 and 7 were obtained respectively when compared to control. Moreover, higher caspase activity was observed in animals' co-adminsitered ASD and MSG.

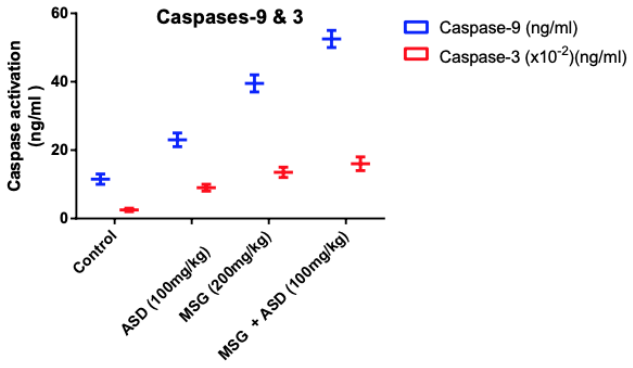

Figure 5:
Effects of alpha stone decoction on caspase activity in a model of MSG–induced hyperplasia in male rats

DISCUSSION

Mitochondria are interesting organelles at the centre of life sustaining and death inducing paths (Bernadi *et al*., 2015). At one end, they are involved in the production of energy via oxidative phosphorylation while at the other they are important determinants of cell death or apoptosis, a physiological process that governs tissue homeostasis, embryonic development and removal of harmful cells (Galuzzi *et al*., 2010) Thus, aberration of the important process is critical to the onset of most diseases (Gupta *et al*., 2009). In view of its critical role in the onset of apoptosis, the mitochondrial permeability transition pore has been an attractive target in diseases where apoptosis is dysregulated (Reed, 1999). The potential safety of the use of MSG has been a matter of debate in recent years. Studies have shown that chronic administration of MSG induces uterine hyperplasia in female rats (Mondal *et al*., 2017) **and** that causes liver inflammation and dysplasia in male wistar rats (Nakanish *et al*.,2008; Sharma *et al*.,2015). We have previously shown that ASD mediates reduction in fibroblast cell count in an MSG-induced model, in this study, we evaluated the effect of ASD on mitochondrial-dependent cell death in a model of MSG-induced hyperplasia was evaluated.

The integrity of the mitochondria isolated from control animals was first evaluated by measuring changes in absorbance of the mitochondrial suspension in the presence of rotenone, an inhibitor of complex I and succinate, the respiratory substrate. Mitochondrial permeability transition was assessed spectrophotometrically for 12 mins at 30 secs interval at 540 nm. Our data showed that the integrity of the mitochondria in untreated animals was not compromised during the process of isolation thus; they were suitable for further use.

We have shown in this study that administration of and alpha stone decoction (100 mg/kgbw) and MSG [200 mg/kg] separately resulted in pore opening by 4.6 and 4.7 folds, respectively while administration of both MSG and ASD produced a synergistic effect on the pore with an inductive fold of 5.0. This suggests that both ASD and MSG is capable of interacting with components of the mPT pore in order to bring about its assemblage and consequently, the opening of the pore. Our data suggest that these substances (MSG and ASD) possess the ability to alter the permeability of the inner mitochondrial membrane by causing the formation of the pore. Given that ASD is a poly herbal formulation, the various components of the plant employed may be more effective than individual components of each plant.

Studies have shown that in mitochondria having leaky inner membrane, the F_0F_1-ATPase work in the reverse direction leading to ATP hydrolysis and increase in inorganic phosphate concentration (Chinopoulos and Adam-Vizi,2010) The observation that ASD enhanced mitochondrial ATPase activity with the release of inorganic phosphate in a model of MSG-induced hyperplasia confirmed that administration of these substances resulted in increase in ATP hydrolysis, thus arresting the bioenergetic function of the organelle. Administration of both ASD and MSG further caused significant enhancement of the F_0F_1 ATPase (3.2 folds) activity when compared with the standard uncoupler, 2, 4 – DNP (5.3 folds). It is obvious that persistent operation of F_0F_1-ATPase in reverse direction to the ATP synthase could deplete the cellular ATP reserves of the cell eventually leading to cell demise (Nicholls, 2000). Although the involvement of mitochondrial ATPase/synthase as one of the mPT pore components is controversial (Bernadi and Di lisa,2015; He *et al*., 2017**),** our data suggest that the mPT pore and the mitochondrial ATPase complex are targets for both ASD and MSG.

Studies have shown that ROS induce a conformational change in the mPT structure which could result in the opening of the pore and sudden increase in the permeability of the inner mitochondria membrane (Zorov, 2014). The observation that administration of ASD elevated levels of malondialdehyde, a product of oxidative damage to lipids, DNA and proteins, when compared to untreated animals in a rat model of MSG–induced hyperplasia suggests that generation of malondialdehyde contributes to the opening of the mPT pore. The increase in MDA levels by administration of MSG emphasizes the role of increased ROS in oxidative damage caused by MSG to several organs as reported by Farombi and Onyema (2006).

The findings that ASD activated caspases 9 and 3 in a rat model of MSG-induced hyperplasia confirm that induction of the pore by ASD resulted in mitochondrial-dependent cell death in the rat model of MSG-induced hyperplasia. Although the the role of MSG in organ dysplasia/ hyperplasia has been reported (Nakanish *et al*., 2008; Oyebode *et al*., 2019), however it appears that aforementioned role is multifaceted, given induction of pore opening with evidence of caspases 9 and 3 activation in this study.

Taken together, the mechanism of alpha stone decoction (ASD) in MSG-induced hyperplasia includes production of ROS in liver mitochondria, decrease in ATP levels and induction of the opening of the mPT pore and activation of caspases -9 and 3. In addition, the possible route by which ASD mediates reduction in fibroids size may be by mitochondrial-mediated apoptosis via induction of mitochondrial permeability transition. However, it seems likely that the role of apoptosis in MSG-induced fibroid development is marginal. Further research is needed to investigate whether induction of cell proliferation by MSG rather than inhibition of apoptosis play a lead role.

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