

## RED PALM OIL: A NATURAL GOOD SAMARITAN FOR SPERM APOPTOSIS?

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### Abstract

Cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP) have been implicated in lipid peroxidation of sperm plasma membranes, DNA damage and apoptosis. This study aimed to investigate the *in vivo* effects of these hydroxides on rat sperm apoptosis, specifically caspase 3/7, and the possible protective effect offered by red palm oil (RPO). Rats ( $n=54$ ) were divided into three groups receiving either standard rat chow (SRC), 2mL RPO (in 25g SRC/day;  $n=18$ ) and 4mL RPO (in 25g SRC/day;  $n=18$ ), respectively. Furthermore, each group was divided into three subgroups ( $n=6$ ). These subgroups consisted of rats injected with saline (control), 10 $\mu$ M cHP or 20 $\mu$ M tbHP. Rats fed with SRC and injected with 10 $\mu$ M of cHP or 20 $\mu$ M of tbHP showed a significant increase ( $P<0.05$ ) in caspase 3/7 activity compared to the control group (injected with 0.5mL saline). On the other hand, animals fed with SRC in addition to 2mL or 4mL of RPO and injected with 10 $\mu$ M of cHP or 20 $\mu$ M of tbHP showed a significant decrease ( $P<0.05$ ) in the production of caspase 3/7 activity compared to those fed with SRC only. It can be concluded that RPO possibly reduces caspase 3/7 activity, thereby, inhibiting apoptosis caused in rat sperm by the *in vivo* induction of hydroperoxide.

### Introduction

Apoptosis, or programmed cell death due to DNA fragmentation, is a distinctive form of eukaryotic cell death characterized by a series of morphological and biochemical changes that result in the elimination of cells from the tissues without eliciting an inflammatory response<sup>[1]</sup>. This genetical-physiological process leads to discard abnormal or damage spermatozoa to ensure cellular homeostasis during spermatogenesis in the form of cell suicide.

Reactive oxygen species (ROS) generated by abnormal sperm can stimulate the process of apoptosis, resulting in the death of sperm. ROS initiates a chain of reactions by activating caspases, ultimately leading to apoptosis<sup>[2]</sup>. When ROS levels are raised pathologically, the process of apoptosis is also initiated in mature sperm. The process of apoptosis is accelerated by ROS-induced DNA damage, which in due course leads to a decline in the sperm count. Oxidative stress, due to excessive generation of ROS, is presumed to cause DNA damage in spermatozoa and has been correlated positively with apoptosis<sup>[3]</sup> and negatively with the fertilisation rate<sup>[4-6]</sup>. Two distinct pathways exist in the initiation of apoptosis. In the extrinsic or receptor-linked apoptotic pathway, the induction of apoptosis occurs via death receptors (cell surface receptors) that transmit apoptotic signals initiated by specific ligands<sup>[7, 8]</sup>. The intrinsic pathway is triggered by stress stimuli, including growth factor deprivation and DNA damage<sup>[9]</sup>. This pathway involves the release of an extrinsic protein, cytochrome c, on the outer surface of the inner mitochondrial membrane of the mitochondria during apoptosis<sup>[7, 9]</sup>. The activation of the apoptosis-signalling pathway occurs in response to regulatory factors such as bcl-2<sup>[10, 11]</sup> and p53<sup>[12]</sup>.

The induction of apoptosis via intrinsic or extrinsic apoptotic pathways result in the activation of an initiator caspase, which activates a cascade of events leading to the activation of effector caspases, responsible for the cleavage of key cellular proteins that lead to the typical morphological changes observed in cells undergoing apoptosis. Caspase-8 and caspase-10 are inhibitor caspases in death receptor-mediated apoptosis, while caspase-9 is the initiator caspase in mitochondrion-dependent apoptosis<sup>[13]</sup>. These pathways differ in one fundamental aspect, i.e., one is external as it is promoted by a series of specific external ligands operating through defined transmembrane receptors and the other is an internal system where activation of the effector enzymes is induced by intracellular changes, involving the mitochondria<sup>[10, 14]</sup>. Despite the difference in the manner of initiation, the extrinsic and intrinsic pathways merge at the level of caspases-3 and 7, and once activated, they cleave intracellular targets, ultimately leading to the manifestation of apoptosis<sup>[15]</sup>.

The supplemental intake of vitamins A, vitamin E and/or vitamin C improved reproductive function in laboratory and farm animals<sup>[16-18]</sup>. *In vitro* administration of vitamin C to patients suffering from congestive heart failure have been shown to suppress apoptosis in endothelial cells<sup>[19]</sup>. It was also reported that the intake of vitamin C improves sperm quality in heavy smokers<sup>[20, 21]</sup> and in male factor infertility patients<sup>[22, 23]</sup>.

Red palm oil (RPO) is the only vegetable oil with a balanced composition of saturated and unsaturated fatty acids both in processed and unprocessed forms<sup>[22]</sup>. RPO contains carotenoids, phosphatides, sterols, tocopherols and trace metals<sup>[22]</sup>, shown to be effective against oxidative stress *in vitro* and *in vivo*<sup>[23]</sup>.

Isong and co-workers<sup>[24]</sup> had shown that RPO exerted effects on reproductive capacity by improving the efficiency of protein biosynthesis or utilization in such a way that was favourable to sex hormone function in rats fed with RPO<sup>[25]</sup>. It is also likely that RPO provided vitamin A, which is known to play a part in reproduction through the synthesis of sexual steroids<sup>[25]</sup>, embryogenesis and spermatogenesis<sup>[26]</sup>. Findings from animal models have highlighted the protective/therapeutic role of caspase inhibitors in many systemic diseases such as cardiac arrest, neurological and rheumatoid diseases or in cases undergoing organ transplantation<sup>[27]</sup>. In this study, we explored the effect of RPO, a natural 'cocktail' of antioxidants, on caspase 3/7 activity in rat sperm apoptosis *in vivo*.

### Materials and Methods

Ethical approval was obtained from the institutional Review Board. Male Wistar rats ( $n=54$ ) aged 10-12 weeks were placed randomly in three groups and fed *ad lib* with standard rat chow (SRC) in an ethical approved animal facility. Group 1 ( $n=18$ ) received no supplement while the food of groups 2 ( $n=18$ ) and 3 ( $n=18$ ) were supplemented with 2mL and 4mL RPO (Carotino SDN BHD Co: 69046-T, Johar-Bahru, Malaysia) in 25g SRC/day, respectively. Each group was divided into 3 subgroups ( $n=6$ ). These subgroups were injected with saline (0.5mL), 10 $\mu$ M cHP (0.5mL, 80% aqueous; Sigma Chemical Co, South Africa) or 20 $\mu$ M tbHP (0.5mL, 70% aqueous; Sigma Chemical Co, South Africa), respectively. The injections were performed daily for 5 consecutive days per week over a period of 60 days in order to target at least one complete cycle of spermatogenesis. Animals were sacrificed and the epididymis were immediately excised and rinsed, followed by gentle macerated in 1.5mL of phosphate buffered saline (PBS; Sigma Chemical Co, South Africa). Sperm cells were retrieved, divided into aliquots and the concentration was adjusted to 2x10<sup>6</sup> cells/mL. The caspase-Glo® 3/7 assay was subsequently performed according to the protocol provided by the manufacturer (Promega, UK).

### Statistical analysis

GraphPad™ PRISM 4 was used for all statistical evaluations and graphical representations. Data are expressed as mean±S.E.M. A one-way analysis of variance (ANOVA) test (with Bonferroni post test if  $P<0.05$ ) was used for statistical analysis. Differences were regarded statistically significant if  $P<0.05$ .

### Results

Caspase 3/7 activity was expressed as Relative Luminescence Units (RLU). Animals injected with 20 $\mu$ M of tbHP (Figure 1) had significantly increased

caspace 3/7 activity in their sperm compared to those injected with saline alone (4298±308.2 vs. 3161±236.5,  $P<0.05$ ). The amount of caspace 3/7 produced by sperm after *in vivo* tbHP administration was significantly lower in rats receiving the 2mL (2803±368.4,  $P<0.05$ ) and 4mL (3021±365.9,  $P<0.05$ ) RPO supplementation to their food when compared to the tbHP group fed only on SRC (Figure 1).

From Figure 2, it is evident that rats injected with 10µM of cHP had a significant increased production of caspace 3/7 in their sperm compared to those injected with saline only (4183±289.6 vs. 3161±236.5,  $P<0.05$ ). Interestingly, the amount of caspace 3/7 produced in the sperm of rats fed with 2mL (2966±306.1,  $P<0.05$ ) or 4mL (3017±333.6,  $P<0.05$ ) supplementation of RPO in addition to cHP injection also decreased significantly when compared to rats receiving SRC only and injected with cHP (4183±289.6,  $P<0.05$ ). RPO supplementation alone did not affect caspace 3/7 activity (data not shown).

**Discussion**

ROS and its role in male infertility have been researched extensively<sup>[28-31]</sup>. Many studies have shown the adverse effects of ROS on the different cellular compartments of spermatozoa, including the DNA. High quantities of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can result in single- and double-strand DNA breaks and apoptosis. Thus, there is a cause to effect relation between apoptosis and DNA damage. This explains the positive correlation between H<sub>2</sub>O<sub>2</sub>, DNA damage and apoptosis<sup>[32]</sup>. H<sub>2</sub>O<sub>2</sub> may trigger other signalling cascades related to apoptosis via oxidation of amino acid residues and other cell constituents.

However, the role of caspases and apoptosis in ejaculated sperm remain unanswered. Caspase, c-jun, p53 and p21 are present in a restricted site for apoptosis (cytoplasmic droplets) in spermatids and immature spermatozoa<sup>[33]</sup>. Inactive and active forms of caspase markers have been detected in human sperm cells<sup>[34, 35]</sup> in both low and high motility fractions of donors and patients. A significant positive correlation has been shown between *in situ*-active caspase 3 in the sperm midpiece and DNA fragmentation in the low motility fractions of patients. This suggests that caspase dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria, and function in the nucleus<sup>[34]</sup>. Mature sperm do not have efficient operative mechanisms for protein synthesis. Both active and inactive forms of caspases (caspase 3) are absent in mature sperm cells<sup>[35]</sup>. They do not show bicarbonate/PKA dependent signs of apoptosis such as fractionation of DNA or mitochondrial inner membrane depolarization, but do show rapid amino phospholipids exposure<sup>[35]</sup>. Paasch and co-workers<sup>[36]</sup> reported that active caspases were present in subpopulations of mature sperm and to a greater extent in sperm from infertile patients. This shows that sperm cells with immature appearance and/or cytoplasmic droplets fail to expose phosphatidylserine (PS) and also shows no phosphotyrosine labeling<sup>[35]</sup>. Alternatively, triggering of PS externalization and DNA fragmentation could be due to activation of other caspases or cellular pathways. It also leaves open the possibility that sperm apoptosis may, to some extent, be caspase independent. It can be seen from our results that despite the *in vivo* long-term hydroperoxide administration, both 2mL and 4mL of RPO supplementation were able to prevent the induction of caspace 3/7 activation and, thus, apoptosis.

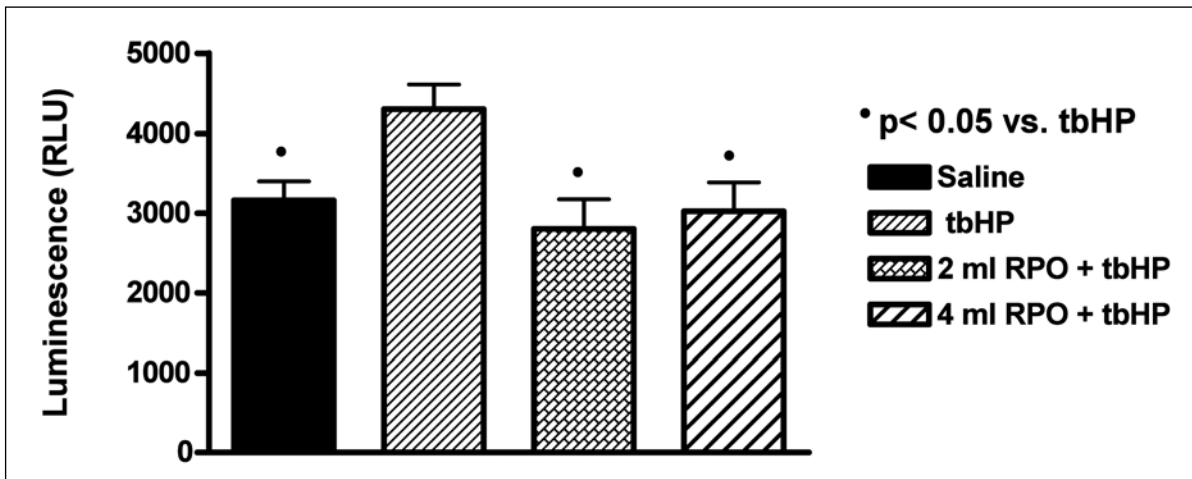


Figure: 1 The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on caspace 3/7 production in rat sperm (n=6 per subgroup).

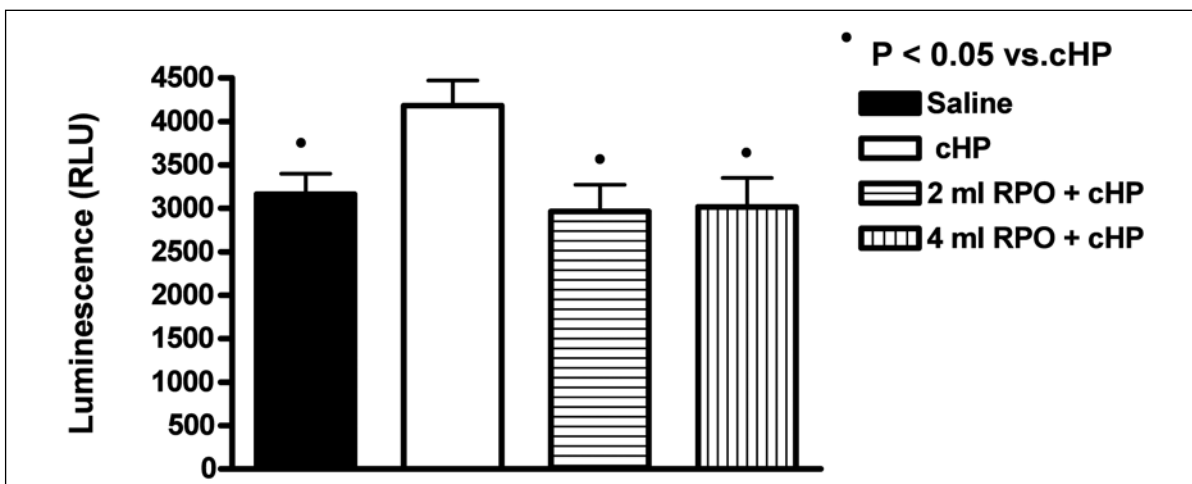


Figure: 2 The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on caspace 3/7 production in rat sperm (n=6 per subgroup).

Since RPO is a natural rich 'cocktail' of antioxidants, it might act by interfering with the activation of the intrinsic or extrinsic apoptotic pathways. Pentikainen *et al.*<sup>[37]</sup> postulated that the expression of the Fas ligand, a known inductor of testicular apoptosis, is down-regulated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Thus, in the seminiferous tubules, germ cell-derived TNF- $\alpha$  may regulate the level of the Fas ligand and thereby control physiological germ cell apoptosis<sup>[37]</sup>. In another study, Ghosh *et al.*<sup>[38]</sup> have shown that the transcription activator nuclear factor NF- $\kappa$ B is a transcription factor expressed in the testis. When activated, NF- $\kappa$ B suppresses apoptosis through the transcriptional activation of genes whose products block apoptosis<sup>[38]</sup>. In normal growing cells, p53 is activated if DNA is damaged. If the DNA has been irreversibly damaged, the cellular p53 may initiate the elimination of programmed cell death and may stop the cell cycle from starting DNA repair<sup>[39]</sup>. From these studies, we postulate that RPO might trigger the production of TNF, NF- $\kappa$ B (or mimic them) or suppress p53 in order to reduce or block apoptosis. Also RPO might block the oxidative stress pathway (caused by H<sub>2</sub>O<sub>2</sub>) that leads to DNA damage and apoptosis. In conclusion, it can be stated that the long term oral supplementation of RPO prevented apoptosis of rat sperm caused by hydroperoxides and can therefore be explored as a useful supplement to protect male germ cells from oxidative stress and subsequent cell death.

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