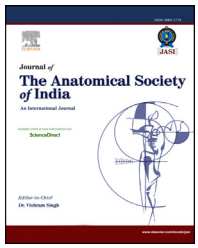
Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jasi



Original Article

Estimation of blood free radical levels in healthy population pre and post yoga

Kuldeep Mohanty [a], *Swetasmita Mishra* [b], *Shiv Basant* [b], *Raj Kumar Yadav* [c], *Rima Dada* [b,*]

[a] *Department of Ophthalmology, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi 110029, India*

[b] *Laboratory for Molecular Reproduction and Genetics, Department of Anatomy, All India Institute of Medical Sciences, New Delhi 110029, India*

[c] *Department of Physiology, All India Institute of Medical Sciences, New Delhi 110029, India*

ARTICLE INFO

Article history:
Received 7 November 2013
Accepted 4 September 2014
Available online 1 October 2014

Keywords:
Reactive oxygen species
Free radicals
Super-oxides
Chemiluminescence
Luminol

ABSTRACT

Introduction: Reactive oxygen species (ROS) serve several physiological functions. At supraphysiological levels they participate in the pathophysiology of many diseases by damaging biomolecules. In several disease conditions and following exposure to insecticides, pesticides and organic pollutants there is oxidative stress. Psychological stress and depression are known to raise cortisol levels and cause oxidative stress. So, this study was planned with the aim to measure and establish baseline ROS levels in whole blood in normal healthy individuals and to investigate the effect of yoga on ROS levels and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels which is an oxidative DNA damage marker.

Method: Whole blood ROS levels was measured in 175 healthy individuals by chemiluminescence method using luminol as a probe. Levels of blood ROS and 8-OHdG were also measured in 50 healthy individuals following a 10 days pretested yoga programme. Statistical analysis was performed using SPSS version 15 software.

Results: The median whole blood ROS value in the healthy individuals was 1266.0 RLU/min/10^4 neutrophil (Minimum – 573.0; Maximum – 2345.0). The mean ROS levels post yoga (1020.81 ± 0.79 RLU/minute/10^4 neutrophil) was significantly lower ($p = 0.024$) as compared to pre yoga (1215.07 ± 0.88 RLU/minute/10^4 neutrophil). The 8-OHdG levels post yoga (9367.57 ± 2709.58 pg/ml) was also significantly lower ($p < 0.041$) as compared to pre yoga (10268.23 ± 3349.71 pg/ml).

Discussion: Baseline ROS value in the whole blood can act as a diagnostic tool in various clinical conditions and exposure to several environmental modulators can disrupt ROS homeostasis and life style intervention like yoga can decrease ROS levels.

* *Corresponding author*. Tel.: +91 (0) 11 26596716.
E-mail addresses: rima_dada@rediffmail.com, rima.dadarima@gmail.com (R. Dada).

http://dx.doi.org/10.1016/j.jasi.2014.09.001

1. Introduction

Molecular oxygen is the terminal oxidant during respiration in aerobic organisms as it is relatively harmless and chemically less reactive. However it has the tendency to be reduced partially to toxic intermediates. These toxic elements are called reactive oxygen species, which are of different types such as i) Singlet oxygen, ii) Hydroperoxyl radical, iii) Superoxide radical, iv) Hydrogen peroxide and v) Hydroxyl radical. These molecules are called reactive oxygen species (ROS). Biomolecules, such as DNA, proteins and lipids are prone to get oxidized by these ROS.[1,2]

Oxygen free radicals are formed in the body from electron transfer to molecular oxygen during the electron transport chain. Superoxide anions which are the precursors of many ROS are formed as a consequence of single electron transfer. Peroxynitrite ($ONOO-$) is formed when O_2^- reacts with nitric oxide. O_2^-, in aqueous medium forms hydrogen peroxide (H_2O_2), by dismutation reaction catalysed by superoxide dismutases (SOD). Hydroxyl radicals ($OH^\bullet$) are generated in the body from H_2O_2 and O_2^- in the presence of copper and iron.[3]

Reactive oxygen species have important roles in several biological processes like cell signalling, regulation of gene transcription and homeostasis.[4] Oxygen radicals are extremely reactive and cytotoxic in all organisms. At high concentrations, they may trigger programmed cell death.[5] Cells have antioxidant systems to maintain ROS levels in the physiological range. Antioxidants inhibit oxidation of other compounds by getting oxidized themselves. There are several enzymatic and nonenzymatic antioxidants in the body, such as glutathione, vitamin C, and vitamin E and enzymes such as catalase, superoxide dismutase and peroxidases, which maintain physiological levels of free radicals.

Physiological levels of reactive oxygen species (ROS) are needed for various normal processes like cellular immunity, sperm function, capacitation, acrosome reaction, proper embryonic development and implantation and gene transcription[6] but when the level of ROS increases beyond the antioxidant capacity of the body it results in a condition known as oxidative stress (OS). OS has been implicated in the pathogenesis of varied human diseases such as atherosclerosis,[7] cancer,[8] diabetes,[9] liver damage,[10] rheumatoid arthritis,[11] systemic lupus erythematosus,[12] cataract,[13] AIDS,[14] inflammatory bowel disease,[15] neurodegenerative disorders,[16] Parkinson's disease,[17] infertility[18] and recurrent spontaneous abortions.[19] Mitochondria are both the source and target of free radicals. Elevated ROS levels damage mitochondrial DNA which further increases free radical production, leading to nuclear DNA damage and hence affects integrity of mitochondrial and nuclear genome.[20,21] Increased ROS levels lead to Ca deregulation causing caspase activation and ultimately cell death.[22,23]

As free radicals are involved in a varied range of disease conditions there should be an easy and direct method to measure free radicals and establish baseline blood ROS levels in normal healthy controls. So we can identify disease conditions in which ROS levels are elevated and could be the underlying mechanism of disease causation. Our laboratory has pioneered in India to establish normal range of seminal ROS levels[21] and also established that higher free radical levels damage both mitochondrial and nuclear DNA and the impact it has on sperm function, embryonic development and foetal well being.[24] We as a part of routine diagnosis measure seminal ROS to evaluate infertile patients. We have found a positive correlation between seminal ROS levels and poor semen quality, nuclear and mitochondrial DNA damage. Seminal ROS levels cannot be considered as indicative of the oxidative status of the whole body but we found a strong positive correlation between seminal and systemic ROS levels. So, blood ROS levels would predict the systemic oxidative status of the body. As blood free radical levels indicate systemic oxidative status of the body, measuring whole blood ROS levels can act as a diagnostic tool for the assessment of clinical conditions that disturb ROS homeostasis and where excess free radicals may be the aetiology of the disease conditions or the disorders may actually raise free radical levels which may lead to sequelae of the disease like autoimmune disorders.

In several disease conditions and due to life style factors (smoking, sedentary life style, exposure to various insecticides and pesticides, electromagnetic radiations, increased consumption of canned foods and obesity) we are increasingly exposed to high free radical levels. If we have baseline free radical levels we can diagnose early cases with oxidative stress and manage by antioxidant therapy and life style interventions or modifications.

Yoga a holistic mind body programme aims at achieving total health-physical, mental, spiritual, social and emotional. Cramer et al performed a meta analysis and reported that yoga does have clinically important effect in cardiovascular diseases and documented that yoga and transcendental meditation can actually regress atherosclerosis and meditation actually is critical in secondary prevention of coronary artery disease.[25] One of the major factors in aetiology of various infectious and inflammatory disorders is oxidative stress. Oxidative stress damages both the mitochondrial and nuclear genome and may be the cause of infertility, recurrent spontaneous abortion and even cancer (Dada R 2014, ISSRF Newsletter). Free radicals target the guanine base in DNA as it has the lowest oxidation potential and causes the generation of a highly mutagenic base 8-OHdG. It results in GC to TA transversions and single and double strand breaks in the DNA. This study highlights the role of yoga in actually decreasing free radical levels and subsequent significant lowering in levels of 8-OHdG. Thus a simple life style intervention can cause significant improvement by causing decline in free radical levels and thus may improve overall health and also prevent and delay onset of several age related and inflammatory disorders.

Clinical interventions involving the administration of compounds that interfere in the free radical homeostasis can disturb the normal cellular oxidative balance and lead to oxidative stress. So, these clinical practices should be accompanied with constant monitoring of the free radical levels before and during the course of the treatment. Baseline ROS levels established in normal healthy individuals would help in the assessment of the effect of these compounds on the normal physiological ROS levels. This study was intended to measure and establish baseline ROS levels in whole blood in

normal healthy individuals and study the effect of yoga on the levels of free radicals and mutagenic oxidative stress marker 8-OHdG.

2. Materials and method

In this pilot study 175 healthy individuals (91 males and 84 females) were enrolled. We recruited 50 healthy volunteers in integral health clinic of Physiology in All India Institute of Medical Sciences, New Delhi, India and they were trained for practicing yoga. Proforma were filled and all subjects were enrolled after taking written informed consent and ethical approval.

2.1. Inclusion criteria

Healthy individuals falling in the age group of 20–40 years with no history of exposure to insecticides/pesticides, non smokers, non alcoholics were enrolled for this study. There was no history of fever, drug intake or change in life style in past 3 months.

2.2. Exclusion criteria

Individuals with recent 3 months history of fever or systemic illness, infection, inflammation and drug intake were excluded from the study.

2.3. Analysis of ROS

Blood was collected by venipuncture. 0.5 ml of the blood was transferred into microcentrifuge tubes wrapped with aluminium foil and containing heparin for ROS estimation and 3.5 ml into EDTA tubes which was sent for the measurement of other blood parameters [Total leucocyte count (TLC), Differential count and Erythrocyte sedimentation rate (ESR)] in order to adjust the chemiluminescence produced during 1 min in relative light units (RLU) per 10^4 neutrophil. To measure the ROS levels in whole blood samples we adopted a luminol enhanced chemiluminescence method.[26,27] Chemiluminescence was recorded using single detector luminometer (Sirius, Berthold Detection Systems GmbH, Pforzheim, Germany). Heparin was preferred over EDTA as an anticoagulant for ROS estimation as we have found in our previous study that being a chelating agent EDTA gives false lower ROS values. Luminol acts as a chemiluminescent probe that reacts with oxygen species produced by neutrophils in the whole blood to produce an excited reaction intermediate. This excited reaction product emits light when it returns back to its ground state. 400 μl of heparinised whole blood was taken in the tube and the readings were measured for 10 min. After 10 min 10 μl of luminol (5-amino-2, 3,-dihydro-1, 4-phthalazinedione; Sigma) was added to the tube and readings were recorded again for 10 min. The reading without luminol was subtracted from the reading with luminol to obtain the original value. Negative control was prepared by adding 10 μL of 5 mM luminol to 400 μL of phosphate buffer saline (PBS) and H_2O_2 was used as positive control. All measurements were done in duplicates and the average values were expressed as RLU/minute/10^4 neutrophil counts as neutrophils are the major producers of ROS in the blood. Since ROS is very transient the samples were assessed for ROS levels immediately after collection. To see the effect of life style interventions like yoga on oxidative stress, levels of blood ROS was measured in 50 healthy individuals trained to practice yoga. Venous blood samples were collected at day 0 (pre yoga) and after 10 days follow up (post yoga practice). ROS was estimated at day 0 and after 10 days by the method described above.

2.4. 8-hydroxy-2-deoxyguanosine (8-OHdG) estimation

Venous blood samples were collected in heparin from each subject. The blood samples were placed on ice and centrifuged at 3500 rpm, 4 °C for 15 min, and the separated plasma were stored at −80 °C. The quantification of 8-OHdG was achieved by using Cayman's EIA kit. Protocol was followed essentially as described by the manufacturer for the quantification of 8-OHdG (Promega).

2.5. Statistical analysis

Statistical analysis was performed using SPSS version 15 and the parameters were compared by Mann–Whitney test. Pearson correlation test was used to find the correlation between parameters and a p value <0.05 was considered significant.

3. Results

There was no statistically significant difference between the age of male group (29.36 ± 5.36 years) and female group (30.04 ± 4.36 years) (p value = 0.50). ROS levels were measured in heparinised whole blood samples. Chemiluminescence was observed every 1 s for 10 min and expressed as RLU/minute/10^4 neutrophil. The samples were done in duplicates and at 2 week intervals and the mean values were considered. Care was taken that each sample was processed under identical conditions immediately after collection The median whole blood ROS level in the healthy individuals was 1266.0 RLU/minute/10^4 neutrophil (Minimum – 573.0; Maximum – 2345.0). The median whole blood ROS level in the male group was 1274.0 RLU/minute/10^4 neutrophil (Minimum – 605.0; Maximum – 2345.0) and in the female group was 1255.0 RLU/minute/10^4 neutrophil (Minimum – 573.0; Maximum – 2313.0). There was no statistically significant difference between the ROS values in males and females (p = 0.66) (Table 1).

Table 1 – Free radical level in male and female groups.

Study Subjects (n = 175)	Males (n = 91)	Females (n = 84)	p value
Age (years) Mean ± SD	29.36 ± 5.36	30.04 ± 4.36	0.50
ROS (RLU/minute/10^4 Neutrophils, Median (range)	1274 (605–2345)	1255 (573–2313)	0.66

Table 2 – Effect of yoga practice on free radical and 8-OHdG levels.

Life style intervention (Yoga)	Pre yoga (Day 0) ($n = 50$)	Post yoga (After 10 days) ($n = 50$)	p value
ROS (RLU/minute/10^4 Neutrophils) Mean ± SD	1215.07 ± 0.88	1020.81 ± 0.79	0.024
8-OHdG (pg/ml) Mean ± SD	10268.23 ± 3349.71	9367.57 ± 2709.58	0.041

The mean ROS levels post yoga (1020.81 ± 0.79 RLU/minute/10^4 neutrophil) was significantly lower ($p = 0.024$) as compared to pre yoga (1215.07 ± 0.88 RLU/minute/10^4 neutrophil). The 8-OHdG levels post yoga (9367.57 ± 2709.58 pg/ml) also declined significantly ($p = 0.041$) as compared to pre yoga (10268.23 ± 3349.71 pg/ml) (Table 2).

4. Discussion

Although remarkable success has been achieved in understanding the sources and mechanism of action of oxidative stress, the role of ROS in many diseases is still to be explored. Though high ROS levels are found in several diseases and it is the chief cause of adverse effect in various diseases (e.g. infertility, recurrent spontaneous abortion[28,29] and glaucoma[19]) but till date whole blood ROS levels are not evaluated in such diseases. If we know baseline levels in normal healthy population only then we can diagnose cases with high ROS levels in various disease conditions and manage and counsel them accordingly. The major problem in measuring ROS is the lack of sensitivity and specificity in the assay methods. Luminol enhanced chemiluminescence method is the most reliable, sensitive and specific method to measure ROS levels. In this pilot study we have established the baseline blood ROS level in healthy individuals using a simple luminol dependent chemiluminescence method. However there are shortcomings of this assay as the ROS levels rapidly decline after collection and thus the samples should be assessed within an hour of collection (preferably within half an hour of collection) and also shows marked variation in different machines for assessing free radicals. Also there is marked fluctuation in ROS levels between 2 readings taken a few days/weeks apart thus newer methodologies need to be devised to assess free radical levels. These are estimation of 8 Isoprostane (8IP) and 8-OHdG which are measure of lipid peroxidation and oxidised guanine base in the DNA respectively. Guanine has the lowest oxidation potential and thus is preferential target of free radicals. In this study we have also assessed levels of this oxidised mutagenic base (8-OHdG).

Blood is the connective tissue that links almost all the systems and organs of the body together. So, oxidative status of the blood can be indicative of the oxidative status of these organs and systems. As this is already established that ROS and oxidative stress is implicated in a varied range of diseases and infections, elevated ROS levels in the blood can be considered as a potential indicator of any infection or pathology or may lead to sequelae of some diseases.[30] To employ blood ROS level as a potential marker of these conditions a baseline blood ROS range needs to be established. In this pilot study we have established baseline ROS level in normal healthy individuals.

It has been reported that humans live in sea of free radicals. Exposure to several manmade chemicals, organic pollutants, endocrine disrupting chemicals like insecticides, pesticides, bisphenol A, phthalates not only disrupt the hypothalmopitutary gonadal axis but also induce oxidative stress.[31] A sedentary life style, fast foods, smoking, excess alcohol intake, electromagnetic radiation, high temperature all result in exposure to increased free radical levels. Thus free radicals can be produced by both exogenous and endogenous sources. Endogenous sources include both acute and chronic infections, inflammations and disorders like obesity. Psychological stress also introduces oxidative stress. Various studies have shown reduction in psychological stress markers like cortisol and inflammation markers like interleukin [IL]-6 and tumour necrosis factor [TNF]-α after yoga intervention.[32,33] Comprehensive life style changes also affect telomere length and telomerase activity. Life style changes (diet, activity, stress management, and social support) have been shown to increase telomere length and decrease telomerase activity.[34] In this study we also found that people who adopted yoga and diet modification showed a significant reduction in the mean free radical levels after 10 days practice of yoga (Table 2). Free radicals cause consistent severe damage to both mitochondrial and nuclear DNA. Since mitochondria have a very basic damage repair mechanism and the mitochondrial DNA are naked molecules with no protective histones and lacking introns, they are the first targets of free radical induced injury. In a previous study from our laboratory[35] we have shown that cases with increased ROS levels had a higher number of non synonymous pathogenic sequence variations in mitochondrial genome compared to cases with normal ROS levels. Also this leads to a vicious cycle as mitochondrial DNA which accumulates these sequence variations (especially in complex I and complex III) become dysfunctional and produce more free radicals and this cycle continues. Thus it is important to identify cases with raised ROS levels (by knowing normal levels) so that they can be managed by adopting a healthy life style (exercise in moderate, yoga, increased intake of fruits and vegetables and meditation). In 2013 Alexander G and associates documented that yoga has ancillary benefits in term of improved physical function, enhanced mental and emotional health, enriched sleep quality and therefore emphasised that it is a health promotion strategy and aids in prevention and management of chronic diseases.[36]

As ROS are involved in several clinical conditions like infections, inflammations and also there are environmental factors that modulate the ROS levels, systemic measures of ROS in the whole blood could act as a potential marker for these clinical conditions and environmental exposures. Measurements of markers for ROS other that direct free radical estimation further substantiate our findings. Yoga a simple life style intervention along with meditation decreased free

radical levels and showed a significant decline in 8-OHdG levels thus should be adopted by both healthy and diseased individuals as yoga not only prevents onset but also delays and retards the progress of disease.

5. Conclusion

In this pilot study we have analysed ROS levels in normal healthy individuals both pre and post yoga. Free radical levels of blood represent the oxidative state of the whole body, hence assessment of ROS levels in the whole blood could serve as a measure of systemic free radical levels which can act as a predictor of several clinical conditions like inflammation and exposure to various environmental modulators of ROS. Short time yoga-based life style intervention in general population reduced blood ROS levels and the levels of 8-OHdG. Thus simple life style interventions can be therapeutic and may profoundly impact health and prevent and retard the progress of several diseases.

Conflicts of interest

All authors have none to declare.

REFERENCES

1. Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod*. 2010;16:3–13.
2. Aggarwal A, Misro MM, Maheshwari A, Sehgal N, Nandan D. Adverse effects associated with persistent stimulation of Leydig cells with hCG in vitro. *Mol Reprod Dev*. 2009;76:1076–1083.
3. Fridovich I. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann N Y Acad Sci*. 1999;893:13–18.
4. Devasagayam TP, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: current status and future prospects. *J Assoc Physicians India*. 2004;52:794–804.
5. Maheshwari A, Misro MM, Aggarwal A, Sharma RK, Nandan D. Pathways involved in testicular germ cell apoptosis induced by H_2O_2 in vitro. *FEBS J*. 2009;276:870–881.
6. Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal*. 2011;14:367–381.
7. Profumo E, Buttari B, Petrone L, et al. Redox imbalance of red blood cells impacts T lymphocyte homeostasis: implication in carotid Atherosclerosis. *Thromb Haemost*. 2011;106:1117–1126.
8. Farinati F, Piciocchi M, Lavezzo E, Bortolami M, Cardin R. Oxidative stress and inducible nitric oxide synthase induction in carcinogenesis. *Dig Dis*. 2010;28:579–584.
9. Victor VM, Rocha M, Herance R, Hernandez-Mijares A. Oxidative stress and mitochondrial dysfunction in type 2 diabetes. *Curr Pharm Des*. 2011;17:3947–3958.
10. Bhogal RH, Curbishley SM, Weston CJ, Adams DH, Afford SC. Reactive oxygen species mediate human hepatocyte injury during hypoxia/reoxygenation. *Liver Transpl*. 2010;16:1303–1313.
11. Winyard PG, Ryan B, Eggleton P, et al. Measurement and meaning of markers of reactive species of oxygen, nitrogen and sulfur in healthy human subjects and patients with inflammatory joint disease. *Biochem Soc Trans*. 2011;39:1226–1232.
12. Shah D, Wanchu A, Bhatnagar A. Interaction between oxidative stress and chemokines: possible pathogenic role in systemic lupus erythematosus and rheumatoid arthritis. *Immunobiology*. 2011;216:1010–1017.
13. Zhang Y, Zhang L, Sun D, Li Z, Wang L, Liu P. Genetic polymorphisms of superoxide dismutases, catalase, and glutathione peroxidase in age-related cataract. *Mol Vis*. 2011;17:2325–2332.
14. Oliveira KF, Cunha DF, Weffort VR. Analysis of serum and supplemented vitamin C and oxidative stress in HIV-infected children and adolescents. *J Pediatr (Rio J)*. 2011;87:517–522.
15. Iborra M, Moret I, Rausell F, et al. Role of oxidative stress and antioxidant enzymes in Crohn's disease. *Biochem Soc Trans*. 2011;39:1102–1106.
16. Jomova K, Vondrakova D, Lawson M, Valko M. Metals, oxidative stress and neurodegenerative disorders. *Mol Cell Biochem*. 2010;345:91–104.
17. Esteves AR, Arduíno DM, Swerdlow RH, Oliveira CR, Cardoso SM. Oxidative stress involvement in alpha-synuclein oligomerization in Parkinson's disease cybrids. *Antioxid Redox Signal*. 2009;11:439–448.
18. Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod*. 2011;26:1628–1640.
19. Venkatesh S, Thilagavathi J, Kumar K, Deka D, Talwar P, Dada R. Cytogenetic, Y chromosome microdeletion, sperm chromatin and oxidative stress analysis in male partners of couples experiencing recurrent spontaneous abortions. *Arch Gynecol Obstet*. 2011;284:1577–1584.
20. Gupta S, Agarwal A, Banerjee J, Alvarez JG. The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: a systematic review. *Obstet Gynecol Surv*. 2007;62:335–347.
21. Venkatesh S, Deecaraman M, Kumar R, Shamsi MB, Dada R. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. *Indian J Med Res*. 2009;129:127–137.
22. Venkatesh S, Shamsi MB, Deka D, Saxena V, Kumar R, Dada R. Clinical implications of oxidative stress & sperm DNA damage in normozoospermic infertile men. *Indian J Med Res*. 2011;134:396–398.
23. Shamsi MB, Venkatesh S, Tanwar M, et al. Comet assay: a prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction. *Indian J Med Res*. 2010;131:675–681.
24. Venkatesh S, Shamsi MB, Dudeja S, Kumar R, Dada R. Reactive oxygen species measurement in neat and washed semen: comparative analysis and its significance in male infertility assessment. *Arch Gynecol Obstet*. 2011;283:121–126.
25. Cramer H, Haller H, Lauche R, Steckhan N, Michalsen A, Dobos G. A systematic review and meta-analysis of yoga for hypertension. *Am J Hypertens*. 2014;27:1146–1151.
26. Verlaan M, Roelofs HM, van-Schaik A, et al. Assessment of oxidative stress in chronic pancreatitis patients. *World J Gastroenterol*. 2006;12:5705–5710.
27. Mohanty K, Mishra S, Pani J, et al. Heparin or EDTA; anticoagulant of choice in free radical estimation? *Oxid Antioxid Med Sci*. 2012;1(1):21. http://dx.doi.org/10.5455/oams.130512.br.001.
28. Tanwar M, Dada T, Sihota R, Dada R. Mitochondrial DNA analysis in primary congenital glaucoma. *Mol Vis*. 2010;16:518–533.

29. Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod*. 2010;25:2415–2426.
30. Aggarwal S, Gross CM, Sharma S, Fineman JR, Black SM. Reactive oxygen species in pulmonary vascular remodeling. *Compr Physiol*. 2013 Jul 1;3(3):1011–1034. http://dx.doi.org/10.1002/cphy.c120024. PubMed PMID: 23897679.
31. Xu H, Yang M, Qiu W, Pan C, Wu M. The impact of endocrine-disrupting chemicals on oxidative stress and innate immune response in zebrafish embryos. *Environ Toxicol Chem*. 2013 Aug;32(8):1793–1799. http://dx.doi.org/10.1002/etc. 2245. Epub 2013 Jun 13. PubMed PMID: 23606268.
32. Yadav RK, Magan D, Mehta N, Sharma R, Mahapatra SC. Efficacy of a short-term yoga-based lifestyle intervention in reducing stress and inflammation: preliminary results. *J Altern Complement Med*. 2012 Jul;18(7):662–667. http://dx.doi.org/10.1089/acm.2011.0265. PubMed PMID: 22830969.
33. Yadav RK, Ray RB, Vempati R, Bijlani RL. Effect of a comprehensive yoga-based lifestyle modification program on lipid peroxidation. *Indian J Physiol Pharmacol*. 2005 Jul-Sep;49(3):358–362. PubMed PMID: 16440857.
34. Ornish D, Lin J, Chan JM, et al. Effect of comprehensive lifestyle changes on telomerase activity and telomere length in men with biopsy-proven low-risk prostate cancer: 5-year follow-up of a descriptive pilot study. *Lancet Oncol*. 2013 Oct;14(11):1112–1120. http://dx.doi.org/10.1016/S1470-2045(13)70366-8. Epub 2013 Sep 17. PubMed PMID: 24051140.
35. Shamsi MB, Kumar R, Bhatt A, et al. Mitochondrial DNA mutations in etiopathogenesis of male infertility. *Indian J Urol*. 2008 Apr;24(2):150–154. PubMed PMID: 19468388; PubMed Central PMCID: PMC2684292.
36. Alexander GK, Innes KE, Selfe TK, Brown CJ. "More than I expected": perceived benefits of yoga practice among older adults at risk for cardiovascular disease. *Complement Ther Med*. 2013 Feb;21(1):14–28. http://dx.doi.org/10.1016/j.ctim.2012.11.001. Epub 2012 Nov 29. PubMed PMID: 23374201; PubMed Central PMCID: PMC3564012.