5.0 METHODS

5.1 PARTICIPANTS

5.1.1 SAMPLE SIZE

Sample size for this study, n = 70 (Experiment group 35 + Control group 35). Sample size was arrived at considering a power (1-β = 0.80), (α = 0.05) and effect size (d = 0.6), from a pilot study conducted at the hospital in S-VYASA.

5.1.2 SELECTION AND SOURCE OF PARTICIPANTS

Subjects identified with T2D (FBS > 126 mg/d), age ranging from 35 to 60 from both genders (Male:33, Female:28), and having no prior yoga experience were enrolled using convenience sampling for this study. All participants of this study were service personnel or their immediate family members from Adugodi Police Quarters, Bangalore, Karnataka, India.

5.1.3 INCLUSION CRITERIA

- Patients identified with T2D
- Fasting Plasma glucose above 126 mg/dL (overnight fasting)
- Age ranging from 35 to 60 from both gender
- Willingness to give written consent for participation in the study

5.1.4 EXCLUSION CRITERIA

Persons identified / diagnosed with,

- Type-1 Diabetes mellitus
- Uncontrolled diabetes, Morbid obesity
- Diagnosed with cardiovascular diseases, cancer
- Diagnosed with neurological or psychological disorders
- Mobility restrictions or inability to do yoga practices
- Alcoholic, or addiction to any forms of drug usage
- Recent major trauma or surgery that would interfere with participation
- Pregnant women
- Persons not versed with English or Kannada or Hindi
- Participation in any interventional study within the past 6 months
- Persons identified with any other serious problem that can confound the outcome

5.1.5 ETHICAL CONSIDERATION

This trial was approved by the Institutional Ethics Committee of S-VYASA (Reference No: RES/IEC-SVYASA/119/2017) and registered at the Clinical Trial Registry of India (Trial Registration No: CTRI/2018/07/014825). Written informed consent was obtained from all the participants before the commencement of the study.

5.2 DESIGN OF THE STUDY

This study followed an assessor-masked randomized controlled trial design. (**Fig 4**)

T2D subjects (n = 61) meeting study criteria were recruited and randomly allocated in a 1:1 ratio using permuted random blocks of variable size (maximum, 6) to Yoga group (31) and Control group (30). An independent researcher from another lab generated a randomization sequence and did the allocation concealment using sequentially numbered, sealed, opaque envelopes. Participants were not blinded from the intervention because of its interactive nature, though for the lab assessments and data analysis, participant identity was masked by coding.

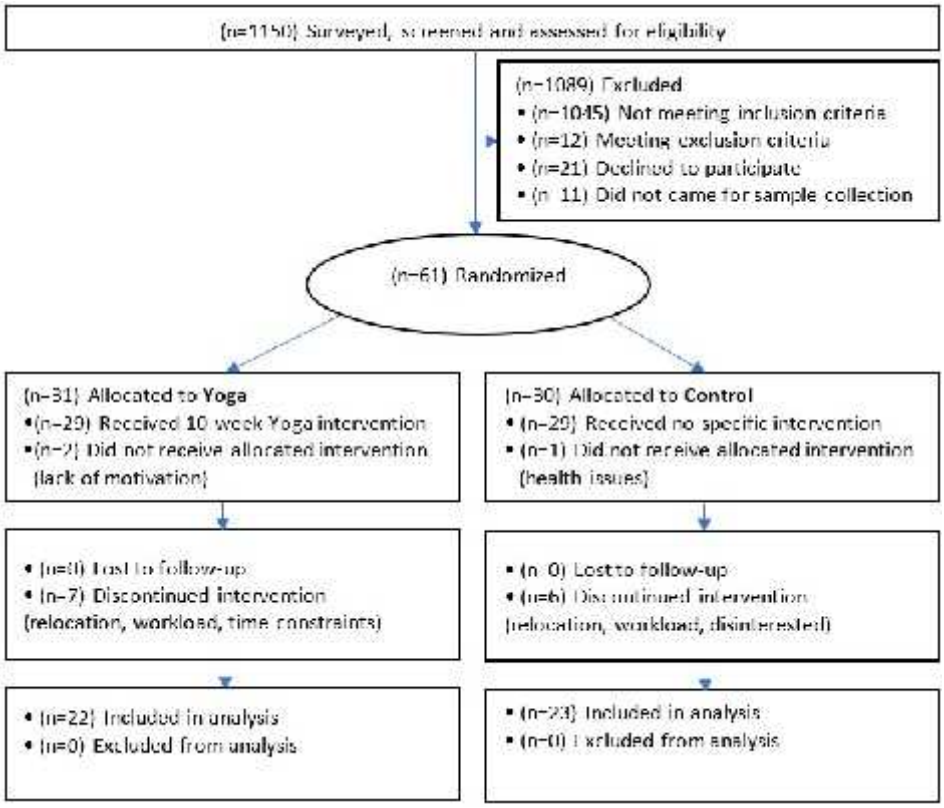

Fig 4. CONSORT Flow Diagram

5.3 VARIABLES STUDIED

Primary outcome measures include Fasting Blood Sugar (FBS) for glycaemic status; Comet assay parameters: Tail Moment (TM) and Olive Tail Moment (OTM) as indices of DNA damage, where TM is defined as the product of the tail length and the fraction of total DNA in the tail (TM = Tail length x Tail DNA%), OTM is defined as the product of distance between the intensity-centroids of the comet head and tail, and the fraction of total DNA in the tail (OTM = Distance between the comet head and tail centroids x Tail DNA%); 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker for oxidative DNA damage; 8-Oxoguanine glycosylase1 (OGG1)protein expression, a primary enzyme responsible for the excision of OS induced DNA damage, for DNA repair activity; and Total Antioxidant Capacity (TAC) for free radical scavenging ability.

Secondary outcome measures include Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL), Triglycerides (TG), Total cholesterol (TC), Creatinine (Cr), Urea, Uric Acid (UA), Albumin (Alb), Total Protein (TP), Systolic and Diastolic Blood pressure (SBP, DBP), Waist-Hip Ratio (WHR), and Body Mass Index (BMI).

5.4 INTERVENTIONS

The Yoga group underwent 10 weeks of yoga sessions including *Asana* (specific postures) and *Pranayama* (specific breathing) practices, 1 hour per day for 4 days a week, by a certified and trained yoga professional at a designated place. On average, a participant in the Yoga group attended 37 hours of instructed yoga practices. This study used an adapted version of the yoga module, developed, validated, and used by AYUSH, Ministry of Health, Govt. of India, for a national level program for T2D management (Nagarathna et al., 2019). Yoga practices were provided as adjuvant therapy to their already existing course of treatment. The

Control group did physical exercises like walking, jogging, and stretching 4 hours/week for 10 weeks, though they were not instructor-led sessions.

5.5 DATA EXTRACTION

LAB PROTOCOL DESCRIPTION

Sample collection and processing:

Venous blood samples (fasting, 10ml) were collected by a trained phlebotomist both at baseline and at the end of 10^{th} week. Blood was drawn into both heparinized and serum vacutainers (BD Vacutainer). Heparinized blood was used to separate peripheral blood mononuclear cells (PBMCs) which was stored at -80°C until further use. Serum was separated from serum vacutainers was also frozen at −80°C untilanalysis.

Anthropometric and Biochemical assessments:

Anthropometric measurements were taken according to standardized procedures (WHO Consultation on Obesity (1997: Geneva et al., 1998). Height, hip and waist circumferences (in cm), Weight (in kg) was measured using digital weighing machine (OmronHN-286, India). Systolic and diastolic blood pressure were measured using digital BP monitor (Omron HEM-7121, India) placed at the upper arm in sitting position. Derived measures like Waist-Hip Ratio (WHR) and Body Mass Index (BMI) were obtained using standard calculations. Same measuring devices were used for both pre and post data collection to ensure consistency in readings. All the biochemical variables; FBS, Lipid profile (LDL, HDL, Triglycerides, Total Cholesterol), Creatinine, Urea, Uric Acid, Albumin, andTotal Protein were measured using automated serum chemistry analyser (Mindray BS390, Mindray Bio-Medical Electronics Co., Ltd, Shenzhen, China).

ALKALINE COMET ASSAY

PBMC isolation and processing for Comet Assay

Peripheral blood samples (10 mL) from T2DM patients in both groups were collected via standard venepuncture into tubes containing EDTA, before and after the yoga intervention period**.** Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich Inc., USA). The PBMC layer was mixed with equal volume of RPMI-1640 media and 10% DMSO before storing at -80^{0}C until further use (Nazarpour R et. al., 2012). The cells were thawed according to Ramachandran et. al, (2012) to maintain maximum viability.

Comet Assay procedure

Alkaline comet assay is a sensitive technique for measuring DNA damage in single cells. It is based on the principle that fragmented or the damaged DNA will migrate out of the cell during agarose gel electrophoresis, which can be visualised as a 'comet tail', while the undamaged DNA that remains within the cell membrane creates the 'comet head'. Here, the length of the tail and % of DNA in the tail will be proportional to the fragmentation, which is indicative of the extent of DNA damage as shown in the image below.

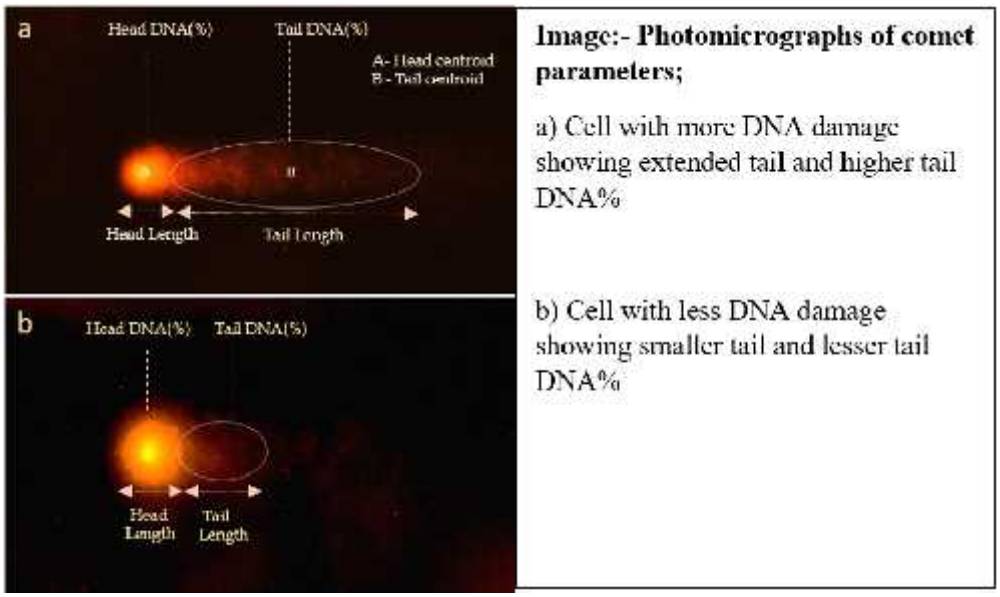

Fig 5. Comet assay images showing DNA damage parameters

In this study, alkaline Comet assay was performed according to Kumar PV et. al.,(Kumar et al., 2015) using PBMCs. Briefly, a freshly prepared suspension of PBMCs (approx. 1×10^6 cells/mL in PBS) was mixed with 0.5% low melting point agarose (HiMedia, Cat No: MB080)and put onto microscope slides pre-coated with a layer of 1.0% normal melting point agarose (NMPA)(HiMedia, Cat No: MB002). The gel layers on the slides were covered with coverslips and were subsequently incubated on ice for 5 min, followed by an additional layer of 1% NMPA and 10min incubation on ice. After removing the coverslips, the slides were immersed in a cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO) for 2hrs at 4°C. Next, the slides were immersed in cold electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 min to allow DNA to denature, followed by electrophoresis at 25 V (1 V/cm, 300 mA) for 30 min. Subsequently, the slides were treated with neutralization buffer (0.4 M Tris–HCl, pH 7.2) for 5 min and washed with cold double distilled water. Slides were stained with 80µL ofethidium bromide (4.6µlof 0.5mM stock/mL distilled water) and examined under a fluorescence microscope (EVOS FLc, Life Technologies, USA). Images were analyzed and scored using OpenComet (v1.3.1) plugin with ImageJ software (v1.52p). On an average, 40 comets from each sample were used for analysis. Tail Moment (TM) and Olive Tail Moment (OTM) were chosen as indicators for DNA damage (Mozaffarieh et al., 2008) and expressed in Arbitrary Units (AU). TM = Tail length × Tail DNA%. OTM = Distance between the comet head and tail centroids x Tail DNA%.

WESTERN BLOTTING

PBMC sample preparation and protein estimation

For western blotting, the PBMC aliquot was thawed on ice and centrifuged at 2500G for 10 min to obtain the pellet. The pellet was further treated with 100µl homogenization buffer (50mM tris, 150mM NaCl, 1M EDTA, 1% Triton-X, 0.1% SDS) along with 1µl of protease inhibitor cocktail and sonicated with probe sonicator (90Hz, 30 sec cycle). The mixture was centrifuged again at 21000G for 10 min to pellet down the cell debris. The supernatant was estimated for protein concentration by Bradford's method (Bradford, 1976) and suitable volume was calculated for loading onto SDS gel.

Protein estimation by Bradford's method

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250**.** Under acidic conditions, the red form of the dye is converted into its blue form, binding to the protein being assayed. Protein-dye complex in solution results in a shift in the absorption maximum of the dye from 465 to 595nm. The absorption is proportional to the amount of protein present.

Procedure

- 20ul of sample, 80ul of deionized water and 1 ml of Bradford reagent were mixed in a vial.
- The reaction was allowed to take place by incubating at room temperature for 10 minutes
- Absorbance of the reaction mixture was measured at 595nm using a spectrophotometer.
- The protein concentration of sample was calculated based on the BSA (bovine serum albumin) standard curve expressed as mg protein/ml serum

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel composition

10%polyacrylamide gel was prepared by mixing the following constituents in the given concentration

RESOLVING GEL (10ml)			**STACKING GEL (5ml)**	
Reagent	**Volume (in ml)**		**Reagent**	**Volume (in ml)**
Deionized water	4.1		Deionized water	3.05
Acrylamide/bis-acrylamide(30%)	3.3		Acrylamide/bis-acrylamide (30%)	0.65
Tris-Hcl (1.5M, pH 8.8)	2.5		Tris-Hcl (0.5M, pH 6.8)	1.25
SDS (10%)	0.1		SDS (10%)	0.05
TEMED	0.01		TEMED	0.005
Ammonium persulfate (APS)	0.032		Ammonium persulfate (APS)	0.05

Electrophoresis procedure

- The resolving gel was poured into the gel casting unit and allowed to polymerize and solidify for 30 minutes.
- A layer of isopropanol was added on top to avoid air bubbles and drying of the gel
- Once solidified, the isopropanol layer is drained out and any traces were removed with distilled water.
- The stacking gel was then poured on top of the resolving gel.
- Comb was inserted into the gel to form the sample wells while the stacking gel was allowed to polymerize for 30 minutes.
- The combs were removed and the gel plate was clamped into the electrophoresis apparatus, immersed in the running buffer and connected to the electrodes (anode and cathode).
- Protein Samples, equivalent to 4-6⇑g protein/well were loaded onto the wells and electrophoresed at 90 Volts, 300mA for 120 minutes

Protein transfer from Gel to Membrane (Blotting)

- The gel-separated proteins were electro transferred (90V, 400mA, 2.5hrs) to PVDF membrane (Cat No.3010040001, Sigma).
- 1X TGM buffer (Tris-25mM; Glycine-192mM; Methanol-10%, pH 8.8) was used to facilitate the protein transfer

Membrane Blocking

- Tris-buffered saline-tween (TBST): 20 mM Tris–Cl, 137mM NaCl, with 0.1% Tween 20 was used as blocking buffer.
- Skim milk powder (5%) was added to the blocking buffer to prepare the blocking solution
- The PVDF membrane after protein transfer was immersed in blocking solution at 4^0C overnight.

Primary Antibody treatment

- The membrane was washed thrice with TBST buffer to remove the blocking solution
- It was then exposed to an anti-OGG1 rabbit polyclonal antibody (Cat No. PAC704Hu01, Cloud-Clone Corp. USA) by immersing the membrane in the antibody solution (Antibody: Blocking solution; 1: 1000) and were incubated at room temperature for 2 hrs
- A shaker (or rocker) was used for gentle agitation of membrane.

Secondary Antibody treatment

- The membrane was then washed thrice with TBST buffer to wash away the unbound antibodies
- Subsequently, membranes were exposed to HRP- linked Caprine anti-rabbit polyclonal antibody (Cat No. SAA544Rb19, Cloud-Clone Corp. USA), at a dilution of 1:5000, at room temperature for 2 hrs in the dark.
- A shaker (or rocker) for gentle agitation of membranes.

Protein detection and imaging

- The membrane was washed thrice with TBST buffer to wash away the unbound antibodies
- The membrane was then treated with a chemiluminescent substrate (Cat No. T7101A, Takara Bio Inc, Japan) specific to HRP conjugate.
- Protein bands were visualized using Syngene G:BOX Chemi-XRQ imaging system (Syngene, UK)with high resolution, cooled camera and GeneSys (v1.4.1.0) software for chemiluminescence applications. β-Actin protein expression was used for normalization.
- Protein bands were analysed using ImageJ software (v1.5.2p, NIH) and expressed in Arbitrary Units (AU).

ANTIOXIDANT CAPACITY

Total Antioxidant Capacity by ABTS method

TAC assay was performed according to the method of Arnao *et al*.,(Arnao et al., 2001) with slight modifications. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)was prepared using 7mM ABTS solution and 2.4 mM potassium persulfate solution. The two solutions were mixed in equal volumes, allowed to react for 14h at room temperature in dark. To prepare working ABTS solution, 1 ml of this was mixed with 60ml of methanol to obtain an absorbance of 0.704 ± 0.01 at 734 nm using a spectrophotometer. Standard curve was prepared using Trolox as reference; with the concentration range 0 -1.5mM. All serum samples were diluted 1:40 before analysis. 190µl of ABTS was added to 10µl of sample/standard. Incubated in dark for 7min at RT and absorbance was measured at 734nm. The antioxidant capacity of the samples was expressed in terms of Trolox Equivalents. All reactions were performed in duplicates.

OXIDATIVE STRESS MEASUREMENT

8-OHdG by ELISA (Enzyme Linked Immuno Sorbent Assay)

8-OHdG (8-hydroxy-2'-deoxyguanosine) in serum was analysed using ELISA kit from Cloud-clone Corp (Cat No. CEA660Ge).This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to 8-OHdG has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labelled 8-OHdG and unlabelled 8-OHdG (Standards or samples) with the pre-coated antibody specific to 8-OHdG. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated.The intensity of the colour developed is inversely proportional to the 8-OHdG concentration and was measured at 450nm using ELISA plate reader (Enspire Multimode Plate Reader, Perkin Elmer, Massachusetts, USA). All reactions were performed in

duplicates. Unknown values were obtained using 5PL standard curve and expressed as pg/ml serum.

Assay Procedure

- Wells for diluted standard, blank and samples were determined. 5 wells for standard points, 1 well for blank.
- 50µL each of dilutions of standard, blank and samples were added into the appropriate wells. Then 50µL of Detection Reagent A was added to each well immediately.
- Plate was covered with a plate sealer. Incubated for 1 hour at 37^{o}C.
- After incubation, the solution in the wells was aspirated and washed by adding 350µL 1X Wash Solution to each well and let it sit for 1-2 minutes. Removed the remaining liquid from all wells completely by inverting and tapping the plate onto absorbent paper. Washing process was repeated 3 times.
- After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. Then inverted the plate and blotted it against absorbent paper.
- 100µL of Detection Reagent B- working solution was added to each well. Incubated for 30 minutes at 37^{o}C after covering it with the plate sealer.
- Repeated the aspiration/wash process for total 5 times.
- 90µL of Substrate Solution was added to each well. Covered with a new Plate sealer. Incubated for 10 - 20 minutes at 37^{o}C. Protected from light using aluminium foil.
- Incubated until the liquid turned blue after the addition of Substrate Solution.
- 50µL of Stop Solution was added to each well, following which the liquid turned yellow.
- Liquid in the wells was mixed by gently tapping the side of the plate to ensure thorough mixing.
- Any drop of water and fingerprint on the bottom of the plate was removed. Thenthe absorbance was measured immediatelyusing a microplate reader at 450nm.

5.6 DATA ANALYSIS

Data were analyzed for outliers and normality and were presented as Mean (SD).

Statistical comparisons (2-sided) between groups were done using Student's Independent t-test for parametric data and Wilcoxon rank-sum test for non-parametric data. Confidence Intervals (CI) were reported with 95% confidence. *P*-value < 0.05 was considered statistically significant. Effect Size (ES) (Cohen's *d*) also was calculated. All tests were done using 'R' statistical software (version 3.6.1).

Mediation Analysis

This study employed a mediation model based on multiple regression to examine and analyze any mediatory role played by oxidative DNA damage (8-OHdG) and DNA repair activity (OGG1) in carrying the effect of yoga on resultant DNA damage (TM). Mediation modeling and analysis were done using Structural Equation Modeling (SEM) technique provided by 'R' statistical package 'lavaan' (version 0.6-5) (Rosseel, 2012).