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MGM Journal of Medical Sciences

1. Aims and Scope

MGM Institute of Health Sciences (Deemed University) recognizes the urgent need for promoting medical education in the country, so that the quality of life for individuals and community could be improved by promoting health, preventing and curing diseases, advancing biomedical and clinical research and educational programs for tomorrow's physicians and scientists. The University is committed to creativity, innovation and excellence in every sphere of its working. The University will transform lives and serve the society by educating, creating knowledge and putting knowledge to work. In this endeavor the University has launched a quarterly peer-reviewed scientific journal 'MGM Journal of Medical Sciences' to encourage investigators to publish their research findings for wider dissemination with the aim of applying those for the benefit of the society.

The newly launched peer-reviewed quarterly journal would cover full spectrum of the specialties in biomedical and clinical research. Its first issue would be released in December 2013. The journal aims to publish articles arising out of original research, specialized topics, review articles, editorials, and description of new diagnostic and therapeutic techniques and technologies. In addition, the journal will include pictorial reviews, letters to the editors, book review, and notices of meetings and courses. In this endeavor, the journal hopes to provide a forum for the stimulation of new developments, clinical practices and research in the field of health and allied sciences. The salient feature of the journal would be to bring out from time to time special issues focusing on specific themes of national relevance including the outcome of scientific meetings, etc. A section would be devoted exclusively to young researchers and students in order to encourage them to publish their innovative ideas and research findings. **In fact, it will be a 'student friendly' journal.**

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Message from the Chairman

It is imperative that medical education takes into account the social, cultural, economic and geographical aspects of the nation. It should address the health problems of each and every individual with diverse socioeconomic status. It is necessary to provide them adequate learning and resource sharing channels to strengthen the health literature and information dissemination mechanism which would ultimately streamline relevant and responsive means to eradicate various diseases. I am extremely happy to learn about the positive initiative taken by the MGM Institute of Health Sciences in bringing out a scientific journal, MGM Journal of Medical Sciences.

Scientific deliberation and exchange of knowledge are the need of the hour. In an age of rapidly changing trends and knowledge that gets updated by the day, it is essential to keep abreast through the print format. It is heartening to note that the MGM Journal of Medical Sciences is available online at its website. Thus, articles and research papers published will move beyond the boundaries of the university, adding a more cosmopolitan feel of the journal.

It is my hope and earnest desire that this medical journal meets the very purpose for which it was evolved for satiating young medical and allied personnel with the scientific bent of mind. I congratulate the Editors-in-Chief, Dr (Lt Gen) Shibban K Kaul, Pro-Vice Chancellor and Dr Chander P Puri, Pro-Vice Chancellor (Research), and the editorial team for their dedicated efforts. I wish the entire team, the best in their efforts towards sustaining and maintaining quality of the issues yet to be published.

Kamal Kishor Kadam
Chairman, MGM Trust

Message from the Vice Chancellor

MGM Institute of Health Sciences (Deemed University) is among the preeminent universities of the country and strives to provide and sustain its high quality in teaching, research and patient care. The university is committed to creativity, innovation and excellence in every sphere of its working. Recognizing the outstanding contributions of the university under the seven major criteria namely: Curriculum aspects; Teaching-learning and evaluation; Research, consultancy and extension; Infrastructure and learning resources; Student mentoring and support including student participation; Governance, leadership and management; and Innovation and best practices, the National Assessment and Accreditation Council (NAAC) of the University Grants Commission had conferred Grade 'A' to the University.

The university has launched a peer-reviewed scientific journal 'MGM Journal of Medical Sciences' to encourage investigators to publish their research findings to allow wider dissemination with the aim of applying those for the benefit of the society. The newly launched journal would cover full spectrum of the specialties in biomedical research. It would include original research articles, review articles, book reviews, case reports, letters to the editor and opinion about research and research management. Special issues would focus on specific themes of national relevance and may also include the outcome of scientific meetings. A section would be exclusively to encourage young researchers and students to publish their innovative ideas and research findings. **It will be a 'student friendly' journal.**

The international editorial board, supported by a panel of reviewers, would ensure that the journal features the high quality papers and is the very best in clinical and laboratory-based research on all aspects of health sciences. We also hope the journal would foster fruitful scientific collaborations. Initially, the journal would be published quarterly, and gradually its frequency would be increased. We invite biomedical scientific fraternity worldwide, who are engaged in research as well as in the application of research results to improve patient care, to get their work acknowledged and highlighted through this platform.

Sudhir N Kadam FRCP (Edin)
Vice Chancellor
MGM Institute of Health Sciences

Editorial

Research: An Integral Part of Education and Health Promotion

Research is a harmonious opera that passionately blends purposeful curiosity and innovative creativity with disciplined process, patient observation and untiring perseverance. Such is the importance of research that without it, humans would still have been scratching each other's back, sitting aside other apes in forests formed over millions of years, literally! Just look around wherever you are and try to identify anything man-made, that does not have its roots in research. In a lighter vein, this search might be far more difficult than research itself, and hence a potentially new topic for research!

Research is therefore an integral to almost every realm of our work in the medical institution whether it is teaching or protecting the health of people or delivering community services. It is directly connected with critical and independent thinking, creativity and more importantly to new discovery. It is the creative ability of the teacher to adopt a holistic approach and utilize his subject knowledge to design course content, develop effective performance tasks and design assessment tools. Creative thinking and experimentation of appropriate research methodologies can result into inventing new ways of learning, which may lead to improved pupil performance, increased motivation, commitment and better behavior.

On the health front, the increased life expectancy at birth of our fellow humans, over the past century, can surely be attributed to advances in medical research. Improvements in awareness about diseases, advances in diagnostic technologies, discovery of new drugs, improvements in drug delivery and surgical techniques have all resulted in an appreciable decrease in mortality and morbidity due to heart attack, stroke, diabetes, breast cancer and even mother-to-child transmission of HIV/AIDS. As our understanding of human biology at the molecular and genetic levels evolves further, one can envision healthcare that would predict our individual susceptibility to diseases, and provide more useful and person-specific tools for preventing diseases.

The larger the university faculty dedicated to research, the better would be the research productivity shown by the university. It is essential to prioritize biomedical research in relation to disease burden and national needs. Public private partnership by bringing together government, academia and industry to build upon strict principles is essential. As far as possible, the outcomes of investment be measurable and address training, scientific consequences, technology creation and economic benefits.

Promoting critical thinking, curiosity for learning, academic medicine, research and innovation within the Mahatma Gandhi Mission Institute of Health Sciences is vital for the future of our students as well as patient care. The MGM Trust recognizes this vital need and commits itself to accomplish this goal. The Trust also appreciates that it requires long periods to move from discovery to competitive product delivery for which long-term, sustainable funding is essential.

Editors-in-Chief

Shibban K Kaul MS MCh FIACS
Pro-Vice Chancellor

Chander P Puri PhD FNASc FAMS
Pro-Vice Chancellor (Research)

Guest Editorial

Perspectives on Medical Education in India

India, the land that Mark Twain called The Cradle of Human Race has also given the world its four major religions; Hinduism, Buddhism, Jainism and Sikhism. The same land gave the world its first university in Takshila (700 BC); and the oldest written texts on our planet—the Vedas. India also developed the knowledge of health and diseases and gave the world, *Ayurveda*, the first School of Medicine. Susruta (800-600 BC), the father of plastic surgery not only healed the sick but trained others in the art of practicing medicine as the present day interns and residents. The original *Susruta Samhita* described the importance of hygiene, and areas of medicine that include midwifery, ophthalmology (removal of cataracts), toxicology, amputation and use of anesthesia, dental extractions, and psychosomatic ailments. The latter conditions are now a significant part of conversation regarding a number of clinical conditions including depression. Thus, medical education in India is solid and deep-rooted, and Indian medical expertise are respected and sought world-wide. Nearly 30% of doctors in the United States are of Indian origin; many educated and trained in India.

Physicians and the practitioners of medicine and healthcare are trusted and have an impactful history. In his now widely read publication 'The 100 – A Ranking of the Most Influential Persons in History' Michael H. Hart notes that 36 such pioneers were Scientists and Innovators (including those engaged in the fields of medicine and healthcare) and only one industrialist. In the United States, the Greentree Gazette reported a few years ago that 37% Americans had great confidence in medicine compared with only 22% and 10% for the White House and the Congress respectively.

The characteristics of contemporary medical education are multi-faceted. It is expected that the delivery of healthcare is patient-centric with an emphasis on evidence-based technologically-empowered clinical practice. The practice of medicine should be based on a larger and broader platform of knowledge and education that is both altruistic and full of compassion. Skills acquired in pursuit of medical education must be applied with the highest level of integrity. We live in a continuously evolving world of technology but a physician still must have core competencies in addition to expertise in special disciplines.

Many new schools of medicine have exemplary curricula. These schools are different than the legacy schools where introduction of new ideas are often met with resistance. Medical education in India would benefit from a curriculum that combines basic education with clinical experience during all four years. In a tradition medical school/college, the first 2 years are dedicated to basic courses related to organs, anatomy and physiological processes, followed by a 2-year experience in clinical environment. Ideally, these two wings of the medical education should be taught simultaneously and, during all 4 years, to make the knowledge and experience fresh and relevant.

Imagine the hallmark contributions of discoveries of basic research to their clinical applications. Delineating the structure of insulin and discovery of DNA were achievements of basic science research but millions of diabetics are living purposeful lives due to availability of genetically synthesized human insulin from bacterial systems. Interdisciplinary research, convergence of basic and clinical experience and combining humanities and sciences can enrich medical education. For example, many patients find it therapeutic and benefit from listening to their favorite music while in critical hospital care.

Personal medicine is emerging on a grand scale in the Western world, whereas in India, it is up to the challenges and opportunities provided by use of gene therapy. Employment of individual gene profiles related to specific diseases (e.g. breast cancer) can assist a physician in selecting either endocrine therapy or other alternatives to save a patient time and money with relatively higher rate of success. Time is ripe when new babies born at the hospitals will be coming home with the parents with a DVD of the child's complete genetic profile.

Stem cell research has emerged as a powerful tool for addressing gene defects and lack or loss of functions that can be recovered by stem cell therapy. Many Indian universities and institutes are now engaged in stem cell and regenerative medicine research which will eventually be a part of most major medical facilities and medicine curricula. Whereas controversies exist as to the source of material for stem cell research; no one discounts the benefits of potential treatment employing this technology.

Telemedicine has recently received favorable attention and resources to address healthcare needs in regions and places not easily accessible (high altitudes, war fronts) or are time-deterrent. Numerous clinics and medical facilities now have options for robotic surgery. Initially made famous for prostate cancer surgery, robots are now in use for cardiac and other serious conditions limiting human error and providing care with precision and minimum invasiveness of the patient.

Medical education must also train physicians to be entrepreneurs and creative in controlling escalating cost of healthcare. In India, unlike the western world, a large number of patients pay cash for healthcare and do not have the traditional medical

insurance. If the noblest of missions for a physician or a medical facility is to heal the sick, then affordability and access to healthcare must be part of medical education conversation and curriculum.

The Mahatma Gandhi Mission (MGM) Trust has taken a bold initiative in establishing MGM Institute of Health Sciences (MGMIHS) under the leadership of Shri Kamal Kishore Kadam to synergize efforts in quality education with the national needs in healthcare sector. Dr Sudhir N Kadam, Vice Chancellor and his team deserve applaud for shaping this institute into a temple of learning and patient care.

With the launch of MGM Journal of Medical Sciences, publication of inspirational and innovative ideas will find consideration and acceptance. This forum will help to strengthen and advance science, technology and medical education in India. Success in aforementioned endeavors will fulfill the aspirations of MGM Trust, its visionary founder and devoted trustees.

Virinder K Moudgil PhD
President and CEO
Lawrence Technological University
Southfield, Michigan, USA

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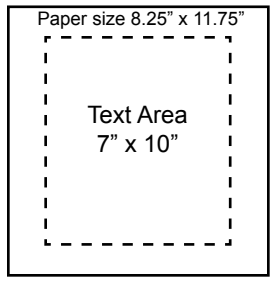

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ORIGINAL ARTICLE

Lipid Peroxidation, Sperm DNA Fragmentation Total Antioxidant Capacity and Semen Quality in Male Infertility

[1]Kavita More, [2]ZG Badade, [3]JG Narshetty, [4] DS Joshi, [5]S Mukherjee, [6]AD Deepak, [7]VZ Badade

ABSTRACT

Infertility is a major clinical problem, affecting people medically and psychosocially. Male factor plays a significant role in about 50% of infertile couples. Recent reports indicate that increasing male infertility could be due to genomic abnormalities. The etiology of sperm DNA damage is multi-factorial but compromised due to nuclear defects, protamine deficiency and oxidative stress. The present study was aimed to evaluate sperm DNA integrity and oxidative stress in infertile men.

The study is prospective, comprises 96 infertile patients and 30 fertile controls. Sperm DNA integrity was assessed by flowcytometry. MDA and TAC were evaluated spectrophotometrically. The percentage of DNA Fragmentation Index and MDA were found to be significantly increased while TAC was significantly decreased in infertile men as compared to control. DFI and MDA were negatively correlated with TAC levels.

Present study indicates significant increases in seminal MDA and sperm DNA damage in infertile men. Seminal MDA was significantly correlated with sperm DNA damage, TAC and standard sperm parameters. The elevated levels of seminal OS observed in these infertile patients could be responsible for poor sperm quality and sperm DNA fragmentation. Hence evaluation of DFI, MDA and TAC can be used for diagnosis, prognosis of male infertility in addition to routine semen parameters to decide the treatment strategies.

Keywords: DNA fragmentation index (DFI), Malondialdehyde (MDA), Oxidative stress (OS), Total antioxidant capacity (TAC).

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INTRODUCTION

Infertility is a major clinical problem, affecting people medically and psychosocially. Its prevalence in Western countries has been estimated as 20% and in India as 15%. Male factor plays a significant role in about 50% of infertile couples.[1-3] Male infertility could be due to genomic abnormalities[4] and assessed by semen analysis. These factors are generally modest predictors of reproductive outcomes that are unable to focus on sperm nuclear and chromatin defects.[5] Men with normal semen analysis can be infertile; sometimes infertile men could not get answer for their decrement semen quality, the cause could be related to abnormal sperm DNA.[6]

The etiology of sperm DNA damage is multi-factorial but compromised due to condensation or nuclear maturity defects, DNA breaks or DNA integrity defects, protamine deficiency, apoptosis and oxidative stress, etc. Oxidative damage to spermatozoa is very common,[7] affecting between 30 and 80% of infertile men, and is due to a number of predisposing factors. There are various sources for generation of oxidative stress in spermatozoa and seminal plasma. First of all, infertile men's semen often contains more morphologically abnormal, immature spermatozoa that have an increased capacity to produce ROS compared with mature spermatozoa. Secondly, sperm plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs), and their cytoplasm and seminal plasma contains low concentrations of scavenging antioxidants, which particularly makes them susceptible to the damage induced by excessive ROS.[8]

Seminal plasma malondialdehyde (MDA) is stable peroxidation product; its estimation is simple and helps to evaluate the effect of peroxidation on sperm. Number of studies has shown that lipid peroxidation affects sperm quality.[9] Antioxidants protect spermatozoa from excess concentration of ROS. The most common antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), α-tocopherol, ascorbic acid, β-carotene, zinc and altogether represent the total antioxidant capacity.[10]

There is evidence to show, infertile men possess substantially more sperm DNA damage than fertile men

and this adversely affect reproductive outcomes.[11] Sperm DNA damage assessment has been recommended as a complementary test in male infertility work-up by some authors. However, it is still unclear whether sperm DNA damage assessment should be introduced as a routine test in infertile men or only applied in selected cases.

In view of etiology of sperm DNA damage by oxidative stress, the objectives of our study was to examine levels of sperm DNA damage and oxidative stress in infertile men and determine the correlation of the sperm DNA damage with seminal MDA and standard sperm parameters.

MATERIALS AND METHODS

The present study was carried out in Department of Biochemistry, Department of Obstetrics and Gynecology, MGM Medical College and Group of Hospitals, Navi Mumbai and National Institute for Research in Reproductive Health, Mumbai. The institutional ethical committee clearance was obtained for the present study.

Thirty male Subjects aged 21 to 45 years, whose partners had conceived within a year and having sperm count ≥ 20 million/ml with motility ≥ 50% in forward progression were selected from general population and considered as fertile (Control Group). Ninety six infertile male (Study Group), referred from Department of Obstetrics and Gynecology, aged 21 to 45 years, without any treatment, whose wives had not conceived after 1 year of regular, unprotected intercourse. The wives of infertile subjects included had no obvious causes of infertility like tubal blockage or ovulation disorders.

In Study Group, patients who had varicocoele, hypogonadism, prolonged illness, leukocytospermia, genital tract infections, cryptorchidism, parotitis, mumps orchitis, tuberculosis, diabetes mellitus, testicular injury, azoospermia due to obstructive causes were excluded from the study. At first clinic attendance, a detail background family and personal history was taken as per written proforma in the form of questionnaire. Detailed physical examination was also done on both husband and wife. The written consent was taken from healthy volunters and infertile males.

Samples were collected by masturbation in wide mouth sterile plastic container after minimum of 3 days of abstinence. After liquefaction, samples were processed by conventional analysis to determine sperm count, sperm motility and sperm morphology according to WHO criteria. On centrifugation, seminal plasma was used for evaluation of lipid peroxidation in terms of malondialdehyde by Satoh K method.[12]

Seminal plasma TAC was determined by using a novel automated method developed by O Erel.[13] In this method, the hydroxyl radical, the most potent radical, is produced via

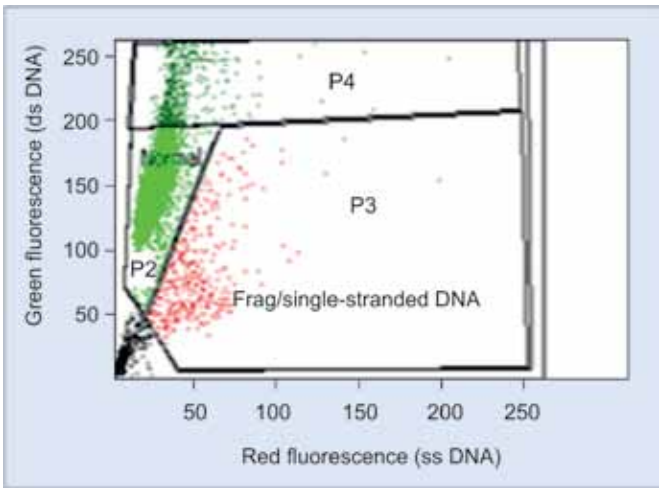


Fig. 1: Representative scatter plot of SCSA analysis of a control subject: (A) Region 1 (P1)—the complete population of sperm being the sum of P2, P3 and P4, (B) Region 2 (P2)—the normal population of sperm with an acceptably low amount of single-stranded DNA, (C) Region 3 (P3)—the population of sperm with an unacceptable amount of fragmented or single-stranded DNA and (D) Region 4 (P4)—the population of sperm exhibiting a high amount of staining associated with double-stranded DNA[40]

Fenton reaction and consequently the colored dianisidinyl radical cations, which are also potent radicals, are produced in the reaction medium of the assay. Antioxidant capacity of the added sample against these colored potent free radical reactions measured the total antioxidant capacity. The results were expressed as millimoles of Trolox equivalent per liter.

Sperm DNA integrity was assessed by the Sperm Chromatin Structure Assay (SCSA) by flow cytometry by Evenson et al method.[14] (FACSDiva Version 6.1.3 flow cytometer (BD Biosciences, USA). The assay is based on the metachromic properties of DNA binding fluorescent dye, acridine orange (AO). AO intercalates with DNA and emits green fluorescence when bound to intact, double strand DNA and red fluorescence when bound to single strand, fragmented DNA.

The SCSA however, quantifies the fluorescent signals by flowcytometry. Upon excitation by laser light, the emitted red and green fluorescent signals from individual cells are detected by photomultiplier tube. The green fluorescence (FL1) was collected through a 515 to 545 nm bandpass filter and the red fluorescence (FL3) was collected through a 650 nm longpass filter. Through a specific SCSA software a scatter plot is created, showing the ratio of green and red sperm. The percentage of red sperm is called DNA fragmentation index, visualized by a histogram.

STATISTICAL ANALYSIS

Statistical analysis of the data was carried out with SPSS, version 16; Data was reported as mean ± SD. The

comparison between two groups was tested by unpaired t-test. A 95% confidence interval was used. $p < 0.05$ was considered statistically significant. Correlation between two continuous outcomes was evaluated using Pearson correlation coefficient.

RESULTS

Results were expressed as mean ± SD for each parameter. Statistically significant differences among infertile and fertile men are indicated in Table 1 (Graphs 1 and 2) along with their significant values. The DFI, MDA and sperm abnormal morphology were significantly increased ($p < 0.001$) whereas TAC, sperm count and sperm motility were significantly decreased ($p < 0.001$) in infertile men as compared to control group.

Correlation coefficient of various parameters indicated in Table 2 along with their significant values. Seminal plasma malondialdehyde and DFI showed negative correlation with sperm count, motility and noted positive correlation with abnormal morphology. Seminal plasma TAC levels were positively correlated with sperm count, motility and have negative correlation with abnormal sperm morphology in infertile men. There was negative correlation between malondialdehyde and DFI with TAC in infertile men. We found the same correlations in control group (Table 3) but most of the correlations are weak and not significant in fertile controls.

DISCUSSION

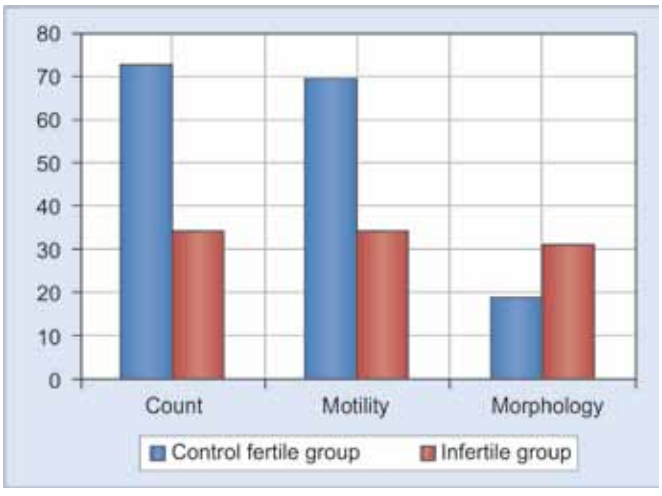
It is established that sperm DNA quality is important in maintaining the reproductive potential of sperm. The fertilizing potential of sperm depends not only on the functional competence of spermatozoa but also on sperm DNA integrity. Sperm DNA integrity has an important role not only for fertilization but also for normal embryo and fetal development, hence any abnormality in sperm DNA integrity could affect any of this process.[6]

Methods of evaluating sperm DNA integrity include the single cell gel electrophoresis assay, i.e. COMET assay, terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL); *in situ* nick translation (NT) and SCSA. We assessed sperm DNA integrity by SCSA and the advantages of the flowcytometric (FCM) technique, such as automation, standardization, objectivity, velocity, precision, reproducibility and statistical robustness.[14]

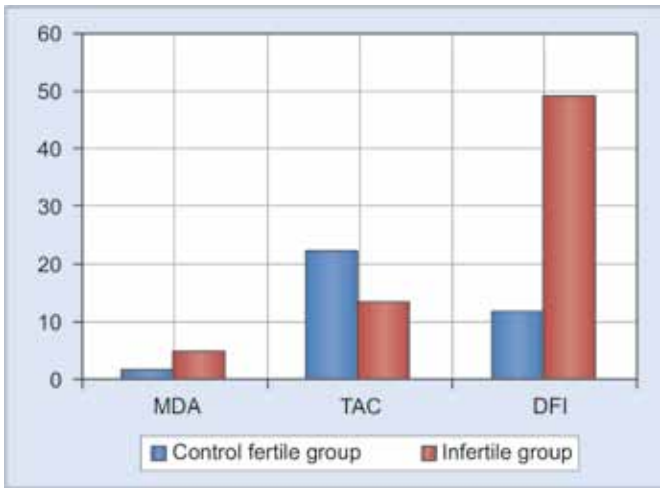
Table 1: Comparison of levels of sperm characteristics, oxidative stress markers (MDA and TAC) and DFI in control group and infertile men

Parameters	*Control group (30) Mean ± SD*	*Study group (96) Mean ± SD*
Sperm count (10^6 millions/ml)	72.74 ± 14.92	34.78 ± 29.55*
Sperm motility (%)	69.16 ± 8.10	34.42 ± 21.45*
Sperm abnormal morphology (%)	18.76 ± 4.74	31.07 ± 13.43*
MDA (nmol/l)	1.71 ± 0.50	5.19 ± 1.31*
TAC (mmol trolox Eq/l)	22.17 ± 2.85	13.79 ± 1.68*
DFI (%)	11.65 ± 3.38	48.90 ± 13.90*

p-value: *Highly significant ($p < 0.001$)



Graph 1: Comparison of sperm count, motility, abnormal sperm morphology of control group and infertile men



Graph 2: Comparison of MDA, TAC and DFI in control group and infertile patients

Table 2: Correlation coefficient of various parameters studied in infertile group

Parameters		*Count*	*Motility*	*Morphology*	*MDA*	*TAC*	*DFI*
Count	r	1	0.120	–0.313**	–0.441**	0.466**	–0.464**
Motility	r	0.120	1	–0.208*	–0.484**	0.284**	–0.485**
Morphology	r	–0.313**	–0.208*	1	0.179	–0.223*	0.143
MDA	r	–0.441**	–0.484**	0.179	1	–0.295**	0.511**
TAC	r	0.466**	0.284**	–0.223*	–0.295**	1	–0.578**

p-value: *Not significant ($p > 0.05$); **Significant ($p < 0.05$)

Table 3: Correlation coefficient of various parameters studied in control subjects

Parameters		*Count*	*Motility*	*Morphology*	*MDA*	*TAC*	*DFI*
Count	r	1	0.132	–0.087	–0.05	0.102	–0.234
Motility	r	0.131	1	–0.306*	–0.20	0.072	–0.089
Morphology	r	–0.087	–0.206	1	0.01	–0.197	0.253
MDA	r	–0.05	–0.200	0.010	1	–0.226	0.165
TAC	r	0.102	0.072	–0.197	–0.226	1	0.048

p-value: *Significant ($p < 0.05$)

Numbers of studies have shown that sperms of infertile men contain more DNA damage than fertile men and may have negative effect on fertility potential and semen quality of these patients. It has been suggested that sperm DNA integrity may be a better predictor of male fertility than routine semen analysis.[14-16] High levels of sperm DNA damage often correlates with poor seminal parameters, such as reduced count, sperm motility and abnormal sperm morphology.[15,17]

In present study, percent DNA fragmentation Index (DFI) was significantly increased in infertile men (48.90 ± 13.90) ($p < 0.001$), and showed significant negative correlation with sperm count ($r = – 464$) ($p < 0.001$), motility ($r = – 485$) ($p < 0.001$) and positively correlated with abnormal sperm morphology ($r = 0.143$). Our results are supported by Evenson DP et a1,[14] Spano et al,[16] Sahel et al.[18]

Saleh et al[18] reported that sperm DNA damage was significantly increased in men with idiopathic and male factor infertility and in men who failed to initiate a pregnancy after assisted reproductive techniques whereas Irvine et al[19] also demonstrated a significant proportion of infertile men have elevated levels of DNA damage in their ejaculated spermatozoa and noted highly significant negative correlations between DNA fragmentation and semen quality, particularly sperm concentration.

Sperm DNA damage may be caused by number of factors. Recently it was suggested that aberrant spermatogenesis could lead to alterations in chromatin packaging and deficiency in protamination which would make sperm DNA more susceptible and vulnerable to variety of stressors, mostly ROS.

Mean levels of MDA in seminal plasma was found to be significantly increased in infertile men as compared to control group. Our results are compatible with Kumar et al[20] Kobayashi et al,[21] Nabil et al,[22] Piyali Das et al,[23] and Badade et al.[24]

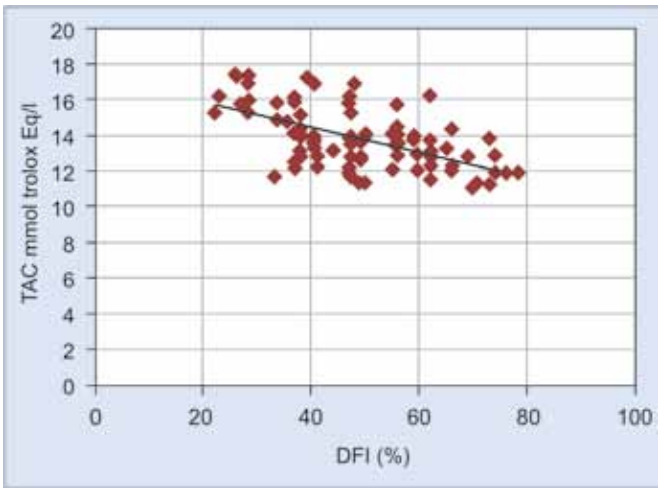
MDA showed a significant negative correlation with sperm count ($r = –0.441$) ($p < 0.001$) and motility ($r = –0.484$) ($p < 0.001$) which was compatible with the findings of Nabil et al,[22] Fraczek et al.[25] We noted positive correlation between sperm abnormal morphology ($r = 0.179$ and MDA which was supported by Shamsi et al.[26]

Recently, it has been reported that percentage of immotile spermatozoa correlate positively with seminal plasma MDA concentrations ($r = 0.50$, $p < 0.01$), while sperm concentration displays a significant negative correlation ($r = –0.63$, $p < 0.001$). On the contrary, a decrement of MDA corresponds to an increase of the pregnancy rate.[27]

Piyali Das et al[23] also found significantly higher levels of MDA in the seminal plasma of the abnormal groups (including oligoasthenoteratozoospermia and asthenoteratozoospermia).

We found high levels of MDA in infertile patients that indicate excessive ROS is responsible for lipid peroxidation of the membrane lipids. Sperm plasma membrane has a high concentration of polyunsaturated fatty acids (PUFAs) which play an important role in ion transport and sperm membrane fluidity, therefore peroxidation of membrane PUFA by excessive ROS disturbs the functions, carried out by the sperm membrane and impairs the fertilizing capacity of spermatozoa.

We found significantly lower seminal TAC activity in infertile men ($p < 0.001$ as compared to control group and showed positive correlation with sperm count ($r = 0.466$) ($p < 0.01$) and sperm motility ($r = 0.284$) ($p < 0.001$) and negative correlation with abnormal sperm morphology



Graph 3: Correlation between DFI and TAC in infertile men (r = –0.578, p < 0.001)

(r = – 0.223) (p < 0.05). Our results are compatible with the Khosrowbeygi A et al,[28] Koca et al,[29] Pasqualotto et al,[30] Pasqualotto et al[31] Moein et al,[32] and Variet et al,[33] Badade ZG et al.[34] We also noted negative correlation of TAC with MDA (r = –0.295) (p < 0.01), DFI (r = –0.578) (p < 0.001) (Graph 3).

Antioxidants are important in maintaining the oxidant-antioxidant balance in tissues which have a significant role in protecting the sperm against peroxidative damage.[35,36] Depressed seminal antioxidant capacity has been implicated in male subfertility. TAC levels have been shown to be lower in the semen of subfertile men as compared with fertile men.[37,38] Nasrin S et al[39] reported DNA damage was significantly correlated with nitric oxide concentration in infertile men and low levels of TAC. Variet et al[33] also demonstrated significant low TAC in male factor subfertility group as compared to fertile control but not reported significant difference in idiopathic infertile group. We found lower TAC levels in infertile patients; this strongly suggests that total antioxidant capacity was insufficient to cope with the excessive amount of ROS.

In this study, elevated levels of sperm DNA damage in subgroups of infertile men may be caused by the high levels of seminal oxidative stress. Another explanation for the link between seminal oxidative stress and sperm DNA damage may be related to defect in spermiogenesis that causes the release of spermatozoa that are immature and have abnormal chromatin structure/high DNA damage and abnormal morphology. Spermatozoa with abnormal morphology have been shown to have a capacity to generate high levels of ROS that, on exceeding critical levels, can cause oxidative stress.

Therefore, results of our study clearly showing that significant elevation of MDA and significantly low levels of TAC in infertile group as compared to fertile men, signify imbalance between oxidants and antioxidants levels which is an indication of increased oxidative stress. This leads to excessive exposure of spermatozoa to oxidative stress that initiate peroxidation of PUFA in sperm plasma membrane. This disrupt sperm membrane fluidity and integrity, as a result of which the spermatozoa lose their competence to participate in the membrane fusion events associated with fertilization, which ultimately causes decreased sperm motility, viability, increased midpiece morphology defects. Further insufficient antioxidants present in seminal plasma makes infertile men's spermatozoa more susceptible to oxidative attack and leads to DNA damage leading to male infertility.

CONCLUSION

Our study indicates significant increases in seminal MDA and sperm DNA damage in infertile men. Seminal MDA was significantly correlated with sperm DNA damage, TAC and standard sperm parameters. The elevated levels of seminal OS observed in these infertile patients could be responsible for poor sperm quality and sperm DNA fragmentation. Hence evaluation of DFI, MDA and TAC can be used for diagnosis, prognosis of male infertility in addition to routine semen parameters to decide the treatment strategies.

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ORIGINAL ARTICLE

Cardioprotective Efficacy of *Bacopa monniera* in Experimental Diabetes Mellitus: Biochemical and Histopathological Assessment

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ABSTRACT

Introduction: Herbs have been used as medicines since ancient times and it has been observed that human body is well suited to herbal remedies. In the present study, the myocardial salvaging effects of *Bacopa monnieri* (L) *Pennell* (*Scrophulariaceae*) (BM), a medicinal herb was evaluated in diabetes mellitus.

Materials and methods: Type II diabetes mellitus was induced chemically in rats using streptozotocin (STZ) (65 mg/kg). Wistar rats were randomly allocated to sham, STZ control and Bm treated groups. Lyophilized hydro-alcoholic extract of Bm (75 mg/kg) was administered once a day orally to the rats for 21 days. On the 22nd day, biochemical parameters (fasting blood sugar and creatinine phosphokinase [(CPK), CPK-MB] and histopathological assessment of myocardium was undertaken to evaluate the cardioprotective efficacy of Bm.

Results: Pretreatment of Bm to experimental rats restored the raised fasting blood sugar levels, CPK, CPK-MB activity and preserved the histopathological architecture of pancreas, heart, liver and kidney as compared to the STZ control group. Bm demonstrated significant cardioprotective effects in the experimental model of diabetic mellitus.

Conclusion: Bm demonstrated significant myocardial salvaging effects in the presence of diabetes mellitus. Histopathological assessment of myocardium shows the cardioprotective effects of Bm in the STZ model of diabetes mellitus.

Keywords: Cardioprotection, Medicinal plant, Streptozotocin, Antidiabetic, Antioxidants.

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, caused by insulin deficiency, often combined with insulin resistance.[1] Oxidative stress due to increased production of free radicals and compromised antioxidant defense mechanisms is known to play a significant role in the pathogenesis of diabetes mellitus.[2]

Diabetes mellitus is an independent risk factor for cardiovascular disease and is associated with increased susceptibility to cardiovascular complications.[3] The majority of diabetes-related deaths arise from cardiovascular complications, such as myocardial infarction, stroke and peripheral vascular disease.[4]

Herbs have been used as medicines since ancient times and it has been observed that human body is well suited to herbal remedies.[5] Therefore, the search for an ideal antidiabetic drug has been extended to medicinal herbs with antidiabetic as well other beneficial effects that may be useful to address the various macrovascular and microvascular complications of diabetes mellitus. These herbs if found effective, may be used as adjuncts to modern conventional medicines. *Bacopa monniera* (Bm), is commonly known as brahmi in Ayurveda, the Indian system of medicine. It has gained world-wide recognition as a memory booster and is used for the treatment of epilepsy and bronchial asthma.[6] The whole plant is therapeutically used and the active ingredients bacosides are mainly responsible for its antioxidant, immunomodulatory and adaptogenic properties.[7,8] The present study was undertaken to evaluate the cardioprotective activity of Bm in the streptozotocin (STZ) model of diabetes mellitus. Further, following STZ induced injury, the safety profile of the medicinal herb was evaluated by studying the histopathological architecture of the liver, kidney, heart and pancreas.

MATERIALS AND METHODS

Experimental Animals

Adult male Wistar rats, 10 to 12 weeks old, weighing 150 to 200 gm were obtained from the Central Animal Facility of MGM Medical College, Navi Mumbai, India, and were

maintained under standard laboratory conditions in the department animal house. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conforms to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research. Rats were kept in polyacrylic cages (38 × 23 × 15 cm) with not more than four animals per cage and housed in an air-conditioned room and were kept under natural light and dark cycles (approximately 14 hours light/10 hours dark) with 60 ± 5% humidity and an ambient temperature of 25 ± 2°C. The animals were allowed free access to standard diet (Amrut Laboratory Animal Feed, Maharashtra) and tap water *ad libitum* and allowed to acclimatize for 1 week before the experiments. Commercial pellet diet contained 24% protein, 5% fat, 4% fiber, 55% carbohydrates, 0.6% calcium, 0.3% phosphorous, 10% moisture and 9% ash w/w. All chemicals were of analytical grade. Streptozotocin was purchased from HIMEDIA, Mumbai chemicals. Double distilled water was used in all biochemical assays. Hydro-alcoholic lyophilized extracts of leaves of *Bacopa monniera* was procured from Dabur Research Foundation, New Delhi, India.

The animals were assigned to the following experimental groups. There were ten animals in each group.

Group 1: Sham Group-Sham Control

Experimental rats were administered 0.9% normal saline orally once a day for 21 days.

Group 2: Streptozotocin Group-STZ Control

The rats of this group were administered 0.9% normal saline orally once a day for 21 days. Diabetes was induced to all the rats of this group by a single dose of STZ (65 mg/kg) intraperitoneally on day 0.

Group 3: Treated Group-Bm-75

Bacopa monniera 75 mg/kg body weight was orally fed once in a day to healthy experimental animals for 21 days. On day 0, diabetes induced to experimental rats by a single dose of STZ (65 mg/kg, IP).

Pilot Study

Dose Selection Study for Bacopa monniera

Previous studies from our laboratory shows that a dose of 75 mg/kg of Bm treatment significantly prevented leakage of myocardial enzyme CPK and preserved the myofiber architecture as compared to the ISP induced myocardial necrosis group. Hence, Bm (75 mg/kg) dose was selected for further evaluation of its cardioprotective effects in the presence of diabetes mellitus.[9]

Streptozotocin Induced Diabetes Mellitus

STZ is a chemical agent that is specifically cytotoxic to beta cells of the pancreas. Diabetes was induced by a single STZ injection (65 mg/kg body wt, i.p. dissolved in 0.01 M citrate buffer, pH 4.5). Serum glucose was estimated (fasting blood sugar > 200 mg/dl) periodically (day 0, 3, 7, 14, 21) from the tail vein to confirm the status of diabetes mellitus.

PARAMETERS EVALUATED IN THE PRESENT STUDY

Estimation of Biochemical Parameters

Blood glucose estimation was done on zero, 3rd, 7th, 14th and 21st day after administrating STZ (65 mg/kg, intraperitoneally) to experimental rats using One Touch Basic Blood Glucose Monitor (Omnitest). The rat blood samples were collected from retro-orbital vein on the 22nd day. Serum was separated by centrifugation at the speed of 300 rpm for 15 minutes for the estimation of creatine phosphokinase (CPK) and CPK-MB. CPK-MB activity was estimated by the method.[10] Average body weight (gm/day) was recorded every week and the change in body weight was calculated after 21 days feeding. At the same time, mortality if any during the 21 days of oral administration of the respective drugs was also monitored.

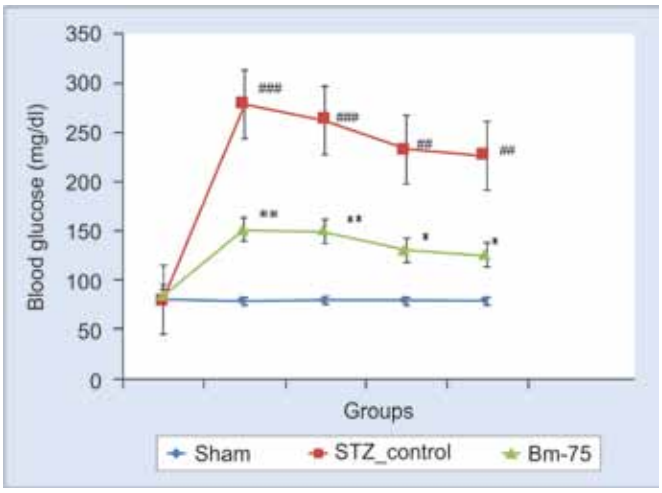
Histopathological Studies

Myocardial, hepatic, renal and pancreatic tissue: at the end of the experiment on the 22nd day the animals were sacrificed. The myocardium, pancreas, liver and kidney were immediately fixed in 10% buffered neutral formalin solution. The tissues was carefully embedded in molten paraffin with the help of metallic blocks, covered with flexible plastic molds and kept under freezing plates to allow the paraffin to solidify. Cross sections (5 μm thick) of the fixed tissues were cut. These sections were stained with hematoxylin and eosin and visualized under light microscope to study the light microscopic architecture of the myocardium, pancreas, liver and kidney. The investigators performing the histological evaluation were blind to biochemical and hemodynamic results and to treatment allocation.

RESULTS

Fasting Blood Glucose (FBS) Levels

The FBS levels in rats of different experimental groups are depicted in Graph 1. Blood glucose levels in sham group rats were found to 80 ± 6.9, 78.1 ± 6.7, 79.4 ± 6.98, 77.8 ± 5.8 and 78.6 ± 6.28 mg/dl at 0, 3, 7, 14, 21 days respectively. Following STZ injection, in the control group rats, blood glucose levels, were elevated from 80.25 ± 7.72 (0 day)



Graph 1: Time course of changes in blood glucose, ###p < 0.001, ##p < 0.01 *vs* sham, **p < 0.01, *p < 0.05 *vs* STZ-control

to 278.75 ± 33.38, 262 ± 26.57, 233.5 ± 9.48 and 226.5 ± 32.02 mg/dl at 0, 3, 7, 14, 21 days respectively. Oral feeding of Bm (75 mg/kg) for 21 days restored the elevated blood glucose levels as compared to the STZ control group. The values were found to 84 ± 2.82, 151.25 ± 24.748, 149.25 ± 17.367, 130.13 ± 5.514 and 125.1 ± 4.65 mg/dl at 0, 3, 7, 14, 21 days respectively.

CPK Activity

In the sham group, the basal levels of CPK were found to be 1779.85 ± 57.57 IU. Following STZ challenge, in the control group rats, CPK was found to be 3483.61 ± 185.4 IU. Treatment with Bm (75 mg kg), prevented this significant rise in CPK values. Values were reduced to 2800.95 ± 164.7 IU.

CPK-MB Activity

In the sham group, a basal level of CPK-MB was found to be 158.4 ± 6.9 IU which increased to 245.7 ± 14.1 IU in the control group, following STZ challenge to rats. Treatment with Bm prevented this significant rise in CPK-MB values. Values reduced to 200.9 ± 18.8 IU.

Body Weight Gain

Weight of sham group rats was found to 187.5 ± 32.73, 198.37 ± 33.16, 215.75 ± 41.73 and 230.625 ± 33.95 gm at 0, 3, 7, 14, 21 days respectively. In the STZ control group rats, weights recorded were significantly less (200 ± 35.25, 176.25 ± 35.53, 156.25 ± 35.12 and 135.62 ± 33.32 gm) respectively at 0, 3, 7, 14, 21 days as compared to sham group. Following oral administration of Bm (75 mg/kg), significant gain in body weight was seen as compared to the STZ control group. The values were found to be 195 ± 24.92, 178.625 ± 30.88, 183.75 ± 34.61 and 200 ± 34.121 gm at 0, 3, 7, 14, 21 days respectively.

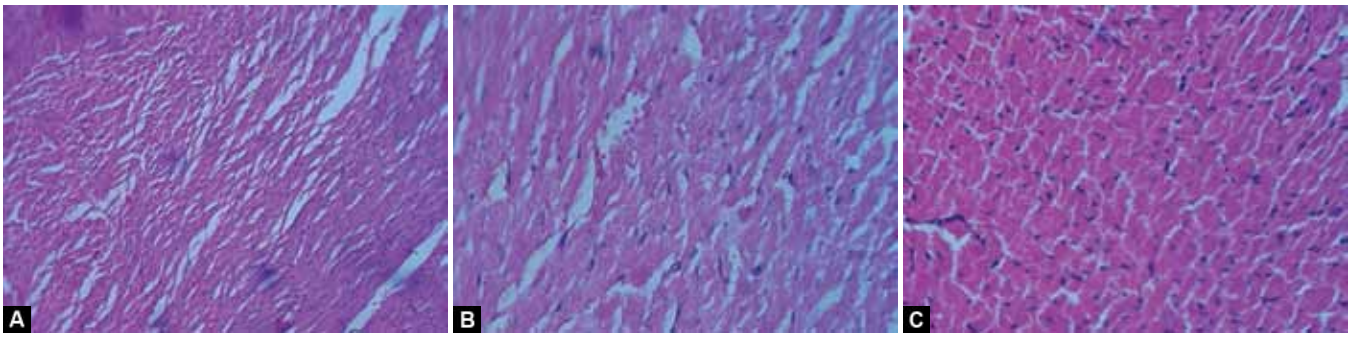
HISTOPATHOLOGICAL ASSESSMENT

Heart

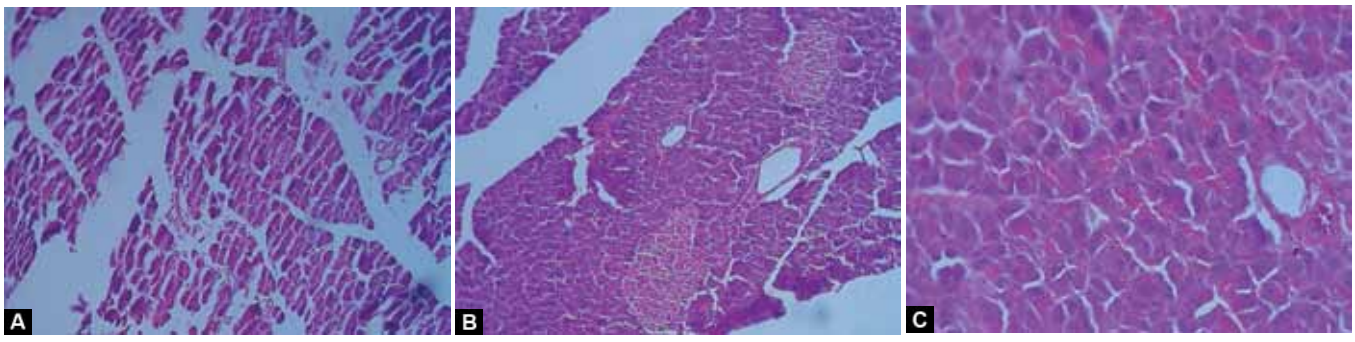
Histopathological assessment of the sham group rat hearts revealed the noninfarcted architecture of the myocardium (Fig. 1A). In contrast, rats subjected to STZ injury, demonstrated marked edema, confluent areas of myonecrosis, separation of myofibrils, congested blood vessels and mild inflammation as compared to the sham group (Fig. 1B). In the Bm treatment group rats, occasional focal myofiber loss, necrosis, edema and inflammation was observed. However, the degree of edema, inflammation and necrosis was less as compared to the STZ control group (Fig. 1C).

Pancreas

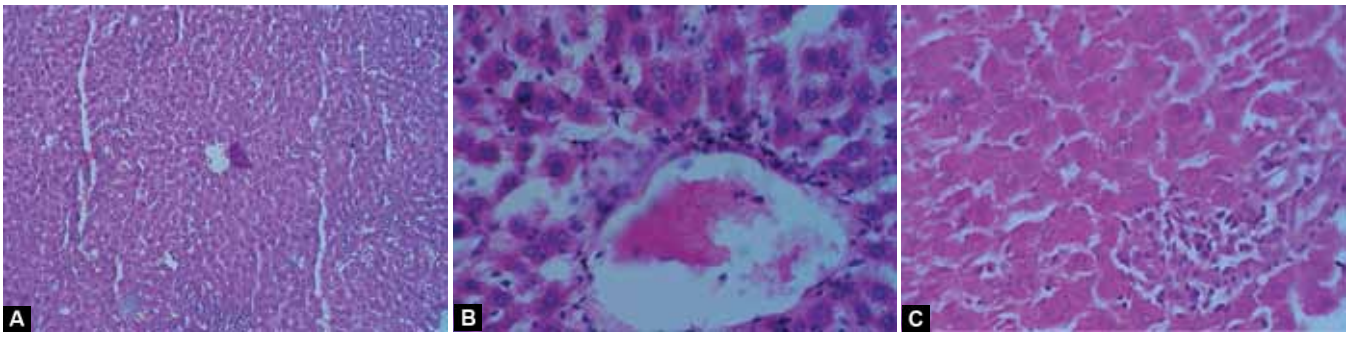
The pancreas of the sham group were characterized by an organized pattern and showed normal architecture of the islets of Langerhans and the beta cells (Fig. 2A). In contrast, in the STZ control group damaged islets of Langerhans and the beta cells with loss of few nucleus and cytoplasm was observed (Fig. 2B). In the Bm treatment group, few nuclear changes were observed though less compared to STZ-control group (Fig. 2C).



Figs 1A to C: (A) The sham group revealed the noninfarcted architecture of the myocardium, (B) contrastively, on histological evaluation, rats' hearts subjected to injury demonstrated marked edema, confluent areas of myonecrosis, separation of myofibrils, congested blood vessels and mild inflammation as compared to the sham group, (C) in the Bm treatment group rats, occasional focal myofiber loss, necrosis, edema and inflammation were observed. However, the degree of edema, inflammation and necrosis was less as compared to the control group



Figs 2A to C: (A) The sham group rat is characterized by an organized pattern and shows normal architecture of the islets of Langerhans and the β cells, (B) in contrast to the sham group, pancreas of the STZ control group rat is characterized by damaged islets of Langerhans and the β cells with loss of few nucleus and cytoplasm and (C) in the Bm treatment group, the pancreas showed no damage in the tissues of islets of Langerhans and the β cells of the rats. However, few nuclear changes were observed though less compared to STZ control group



Figs 3A to C: (A) Sham group shows the normal architecture of central veins, periportal veins and hepatocytes, (B) in contrast to the sham group, the STZ control group rat liver showed degeneration and scattered necrotic cells, multifocal mild degree portal lymphocytic infiltration, swollen cytoplasmic hydropic and microvescicular vacuoles and (C) however, treatment with Bm decreased the granular degeneration in diabetic liver as compared to STZ control group. No congestion in central veins was observed. Periportal inflammation and hepatocytes degeneration were less as compared to the STZ control group

Liver

Histopathological assessment of liver in sham group showed the normal architecture of central veins, periportal veins and hepatocytes (Fig. 3A). In contrast to the sham group, the STZ control group showed degeneration and scattered necrotic cells, multifocal mild degree portal lymphocytic infiltration, swollen cytoplasmic hydropic and microvescicular vacuoles (Fig. 3B). However, treatment with Bm decreased the periportal inflammation and hepatocytes granular degeneration in diabetic liver as compared to STZ control group. Also no significant congestion in central veins was observed (Fig. 3C).

Kidney

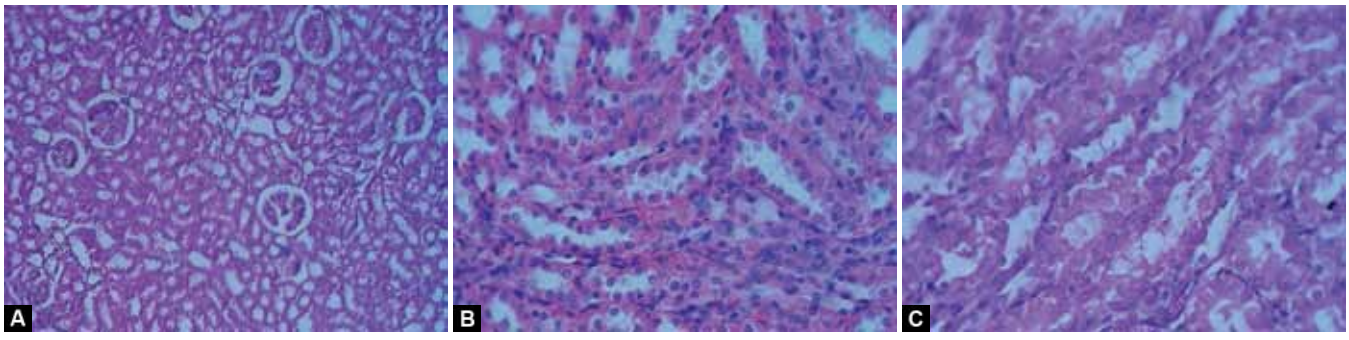
Histopathology of kidney in sham group showed the normal structure of the kidneys. There was absence of congestion of glomerular blood vessels, tubular necrosis, inflammation, cloudy degeneration (Fig. 4A). In contrast, on histological evaluation, rat's kidney of the STZ control group showed congestion of glomerular blood vessels, tubular necrosis, inflammation and cloudy degeneration as compared to the sham group (Fig. 4B). The treatment with Bm showed the congestion of glomerular blood vessels, tubular necrosis, inflammation, cloudy degeneration but it was less as compared to the STZ control group (Fig. 4C).

DISCUSSION

Diabetes mellitus, a metabolic disorder, is characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins with an increased risk of complication of vascular diseases.[4] Type 2 diabetes mellitus is an independent risk factor associated with increased susceptibility to cardiovascular complications, such as ischemic heart disease, myocardial infarction, stroke and peripheral vascular disease.[11,12]

A multifactorial approach for the management of diabetes mellitus addressing its microvascular and macrovascular complications is the need of the hour. There is a large subset of diabetic patients with underlying cardiovascular morbidity.[4] Therefore an anti-diabetic drug with additional therapeutic benefits against cardiovascular complications may be beneficial in this group of diabetic patients.

Medicinal plants have been observed to possess numerous activities such as hypolipidemic, antiplatelet, antithrombotic, cardioprotective, anticoagulant and hypoglycemic



Figs 4A to C: (A) Sham group showed that normal structure of the kidneys. There was no congestion of glomerular blood vessels, tubular necrosis, inflammation, cloudy degeneration, (B) contrastively, on histological evaluation, rats kidney of the STZ control group showed congestion of glomerular blood vessels, tubular necrosis, inflammation and cloudy degeneration as compared to the sham group and (C) the treatment with Bm showed the congestion of glomerular blood vessels, tubular necrosis, inflammation, cloudy degeneration but it was less as compared to the STZ control group

activities with regard to addressing the macrovascular and microvascular complications of diabetes mellitus.[9] Thus, the use of plant derived agents represents an important and novel therapeutic target and strategy for the treatment of diabetes with coexisting complications. With this point of view, the study was designed.

STZ is a chemical agent that is specifically cytotoxic to beta-cells of the pancreas. STZ induces partial destruction of beta cells within 24 hours, thereby triggering an inflammatory process leading to macrophage and subsequent lymphocyte infiltration, which is followed by the onset of insulin deficiency results in hyperglycemia rather than insulin resistance. Several studies have demonstrated that STZ (65 mg/kg) induces diabetes mellitus in experimental rats.[13]

The present study results concur with the previous studies by various authors.[14] Diabetes was induced by a single intraperitoneal STZ injection (65 mg/kg body wt). Following STZ injection, a raised fasting blood glucose levels were observed 72 hours after the injection and maintained throughout the experimental duration; confirming the presence of diabetes in the STZ control group. In the Bm (75 mg/kg) treated group, a significant hypoglycemic activity as indicated by a decline in the fasting blood glucose levels was seen as compared to the STZ control group. A negative correlation between rise in fasting blood sugar and gain in body weight was observed in the experimental groups.

Diabetes also induced adverse cardiovascular changes in the rats as indicated by an elevated serum CPK activity of STZ control rats. The leakage of CPK from the myocardium tissue with concomitant rise in the plasma is considered as a marker of myocardial injury.[15,16] Leakage of this enzyme is a strong evidence for loss of sarcolemmal integrity due to STZ induced injury to the myocardial cells. In the STZ control group, a significant elevation in the serum CPK, CPK-MB and histopathological assessment of the myocardium confirmed the myocardial injury.

The antihyperglycemic activity of Bm was observed in the STZ model of diabetes mellitus. In addition to its beneficial effect on serum blood glucose, the cardioprotective effect of Bm was also evaluated in the present study. Several authors have previously documented the cardioprotective effects of Bm.[9,17,18] Nandave et al 2007[17] demonstrated that the lyophilized hydro-alcoholic extract obtained from Bm, at doses of 100, 150 and 200 mg/kg, provides significant cardioprotection against ISP-induced myocardial necrosis in rats. Bm (150 mg/kg) produced maximum cardioprotection as evidenced by significant restoration of endogenous antioxidants, CK-MB isoenzyme activities and decrease in malonaldialdehyde levels. The cardioprotective effects of Bm at the doses 25, 75 and 150 mg/kg in the ISP model of myocardial necrosis was studied previously in our laboratory.[9,19]

However, this is the first study further exploring the cardioprotective effects of Bm in the presence of diabetes mellitus. In the present study, Bm (75 mg/kg) treatment significantly preserved the myocardial enzyme CPK and myofiber architecture as compared to the STZ control group. Biochemical and histopathological evaluation confirmed the myocardial salvaging effects of Bm (75 mg/kg) in the presence of diabetes mellitus. In addition to the favorable effect of Bm on the myocardial-architecture, Bm treatment also preserved the histopathological structure of the hepatic, renal and pancreatic tissue. Therefore, Bm (75 mg/kg) is a safe efficacious cardioprotective medicinal herb.

Nature has been a source of medicines for thousands of years and plant-derived products continue to play an essential role in the primary healthcare of the world's population.[20] Bm, a medicinal herb traditionally used have been evaluated scientifically in the present study with an aim to define the role of these agents in limiting the deleterious effects of STZ induced diabetes mellitus. The study provides preliminary scientific data to further evaluate Bm as a

promising antidiabetic herb with potential cardiovascular benefits.

CONCLUSION

Bm demonstrated significant myocardial salvaging effects in the presence of diabetes mellitus. Histopathological assessment of the myocardium and CPK levels confirmed the cardioprotective effects of Bm (75 mg/kg) in the STZ model of diabetes mellitus. In addition, Bm (75 mg/kg) pretreatment was safe to the vital organs as it preserved the histopathological structure of the hepatic, renal and pancreatic tissues.

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ORIGINAL ARTICLE

Toxicity Assessment of Silver Nanoparticles in Zebrafish Embryos

[1]Mansee Thakur, [2]Navami Dayal, [3]Girish Pai, [4]Dattatraya Joshi

ABSTRACT

Genotoxic effects of silver nanoparticles (Ag-NP) in a vertebrate model system were investigated.

Effects and accumulation patterns of silver nanoparticles were studied using zebrafish embryos. Nanoparticles of silver were synthesized by chemical reduction of silver nitrate, using sodium borohydride as reducing agent and polyvinyl pyrolidene as a stabilizer. These nanoparticles were characterized by UV/Vis spectrophotometer (absorption spectra), Transmission electron microscopy and were found to have the size range of 4 to 10 nm. Evaluation of cytotoxicity was carried out at various concentrations to obtain the LD_{50} value. Dose dependent decrease in percent viability was observed on exposure of embryos to different concentrations of silver nanoparticles with LD_{50} of 1.0 µg/ml. The results indicate that silver nanoparticles induce a dose-dependent toxicity in embryos and abrogate normal development.

Keywords: Zebrafish embryos, Silver nanoparticles, 4 to 10 nm, TEM, UV/Vis spectrophotometer.

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INTRODUCTION

Nanotechnology is rapidly growing with nanoparticles produced and utilized in a wide range of commercial products throughout the world. Thus, silver nanoparticles (Ag-NP) are used in electronics, biosensing, clothing, food industry, paints, sunscreens, cosmetics and medical devices. These ever increasing range of applications, however, increase human exposure and with potential risk related to their short- and long-term toxicity.[1] Nanoparticles are particularly promising because of their ease of synthesis in various sizes and shapes and the potential for subsequent conjugation with peptides and proteins for targeted drug delivery. Details regarding the interactions of nanoparticles and biological systems in most of these cases are as yet not understood. Therefore, for the safe application of nanoparticles in therapy and drug delivery, information regarding their bioaccumulation and associated local or systemic toxicity is necessary. Information on the biological fate of nanoparticles including distribution, accumulation, metabolism and organ specific toxicity is still scatchy.[2]

There is need for low-cost, high-throughput animal models in some fields of biomedical research, such as drug screening and toxicity assessment.[3,4] The zebrafish is emerging as one such model.[3] Zebrafish are vertebrate organisms that are attracting increasing interest for preclinical drug discovery applications. Zebrafish embryos develop most of the major organ systems, including the cardiovascular, nervous and digestive systems, in less than 1 week. Additional characteristics that make them advantageous for compound screening are their small size, transparency and ability to absorb compounds through water.[5] Genomic sequencing has shown extensive homology between zebrafish and other vertebrate species (including humans), and some aspects of brain patterning, structure and function are conserved.[6-9] They are being used as model systems for various human diseases.

Current dogma states that size and/or surface area is the critical physicochemical property responsible for inducing nanoparticle toxicity.[10-12] Surface coatings functionalize, stabilize nanoparticles and influence the toxic profile and biocompatibility. The effects of surface chemistry or composition should not be overlooked as partial surface composition coupled with a specific size of the nanomaterial is accountable for the observed toxic effects.[8] In this study, simple, conventional chemical method has been used to prepare silver nanoparticles. Results from this study could be useful in the understanding of Ag nanotoxicity and suggest the least effective concentration that could possibly be used in various medicinal and environmental applications.

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MATERIALS AND METHODS

Synthesis of Silver Nanoparticles

Synthesis of silver nanoparticles prepared in the Department of Biotechnology, MGM Medical College, Navi Mumbai, The conventional method for synthesis of metallic nanoparticles, and the reduction of dissolved silver salts ($AgNO_3$) with sodium borohydride as a reducer was used. All reagents used were of analytical grade and solutions of reacting materials were prepared in double distilled water. A 10 ml volume of 1.0 mM silver nitrate was added drop wise (about 1 drop second) to 30 ml of chilled 2.0 mM sodium borohydride solution. The reaction mixture was stirred vigorously on a magnetic stir plate. The solution turned light yellow after the addition of 2 ml of silver nitrate and a brighter yellow when all of the silver nitrate had been added. The color change of the solution indicates the formation of silver nanoparticles. The reduced silver nanoparticles were monitored in UV–Vis spectrophotometer.[13]

Characterization

UV–Vis Spectroscopy

The formation of silver nanoparticles was followed by scanning the solution containing nanoparticles at the wavelength ranged from 300 to 600 nm using double beam UV/Vis spectrophotometer (Thermo Scientific, Evolution 201 series).

Transmission Electron Microscopy (TEM)

The analysis of synthesized silver was carried out on the film coated drop of nanoparticles employing transmission electron microscopy (Philip, Model No. CM200, Operating voltages: 20 to 200 kV resolution 24 Å)

Breeding of Zebrafish

Breeding of zebrafishes is done in order to obtain embryos. The day to night cycle was controlled with an automatic timer (14 hr light/10 hr dark), temperature was maintained at 28.5°C and pH of water was kept neutral. A day before the embryos are needed, the fishes were transferred to a separate breeding chamber in the ratio of one male per two females. Generally, this ratio is opted for maximal embryo production. Embryos obtained the next day were collected and further subjected to exposure at various concentrations of silver nanoparticles.[14]

Exposure of Embryos at Various Concentrations of Silver Nanoparticles

Aqueous solution of silver nitrate ($AgNO_3$) taken as source of ionic silver in addition to the synthesized silver (Ag) nanoparticles. Content of silver in original solution was estimated to be 107 µg/ml and 58.4 µg/ml for ionic silver and Ag nanoparticles respectively. The embryos were exposed to 0.05 µg/ml, 0.3 µg/ml, 0.5 µg/ml, 1.0 µg/ml and 2.0 µg/ml of silver nanoparticles. Study was carried out by exposing zebrafish embryos (n = 20) to each concentration having three replicates per concentration. The embryos were exposed at around 4 hours post fertilization (hpf) and were monitored at different time intervals of 6 hpf, 24 hpf and 48 hpf.

Statistical Analysis

All experiments were repeated at least three times. The results were represented as means with calculated standard deviation and standard error.

RESULTS AND DISCUSSION

UV/Vis Spectroscopy Analysis

UV-visible spectroscopy is a simple method to characterize the size of nanoparticles. By estimating the peak with width at half maxima of the curve; the size of the nanoparticles can be obtained. The distinctive colors of silver nanoparticles are due to a phenomenon known as Plasmon absorbance. Incident light creates oscillations in conduction electrons on the surface of the nanoparticles and electromagnetic radiation is absorbed. The wavelength of the Plasmon absorption maximum in a given solvent can be used to estimate the particle size. Figure 1A represents chemically synthesized Ag nanoparticles having UV/Vis spectra with maxima at around 400 nm. This corresponds to mean diameter of 10 nm.

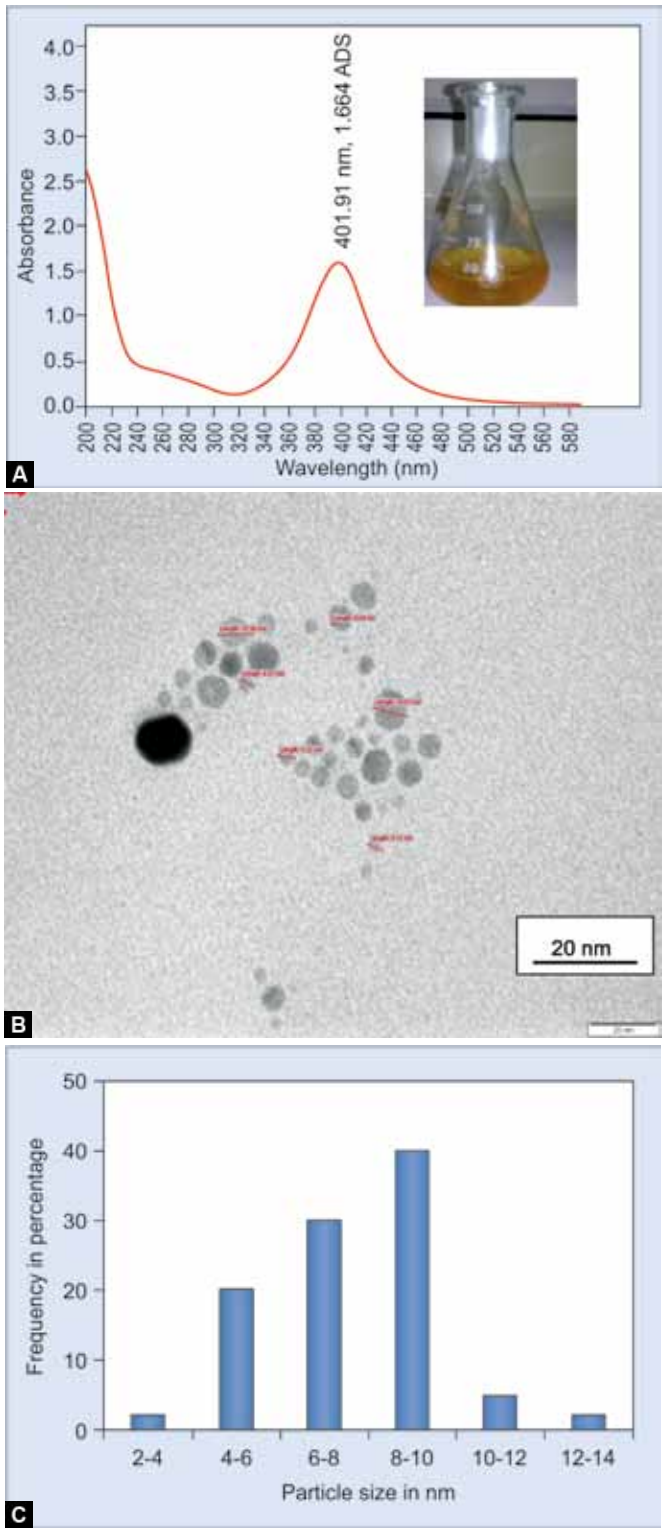
Transmission Electron Microscopy Analysis

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film or to be detected by a sensor such as a CCD camera. Figures 1B and C represents TEM micrograph of Ag nanoparticle and particle size distribution plot.

The size distribution of the nanoparticle (Fig. 1C) indicates that the particle synthesized are within the range of 4 to 10 nm in diameter.

Evaluation of Zebrafish Embryos exposed to Ionic Silver and Ag Nanoparticles

The concentration of ionic silver ($AgNO_3$ solution) measured by Atomic Emission Spectroscopy (AAS) was found to



Figs 1A to C: (A) UV/V is spectrum of AgNPs: absorbance plotted as a function of wavelength (maxima at 400 nm), (B) transmission electron micrograph of SNP and bar indicates 4 to 10 nm and (C) size distribution of SNP. Particle size distribution plot

be 107 µg/ml. The concentration of silver nanoparticles synthesized by chemical reaction on the other hand was found to be 58.4l µg/ml**.**

The embryos were evaluated for toxic adverse effects caused particularly by nanoparticles of small size range, i.e. 4 to 10 nm. They were monitored for delay in development, morphological defect and survival rate assessed during a period of 0 to 72 hpf. Figure 2A indicates control embryos for ionic silver and Ag nanoparticles. Based on the above parameters, an overall LD_{50} value of 0.05 µg/ml and 1.0 µg/ml for ionic silver and Ag nanoparticles respectively was calculated (Figs 2B and C). Table 1 represents summarized data showing average viability of hatched embryos at 72 hpf for exposure toward ionic silver and silver nanoparticles.

Exposure of Embryo against Ionic Silver ($AgNO_3$) and Silver Nanoparticles

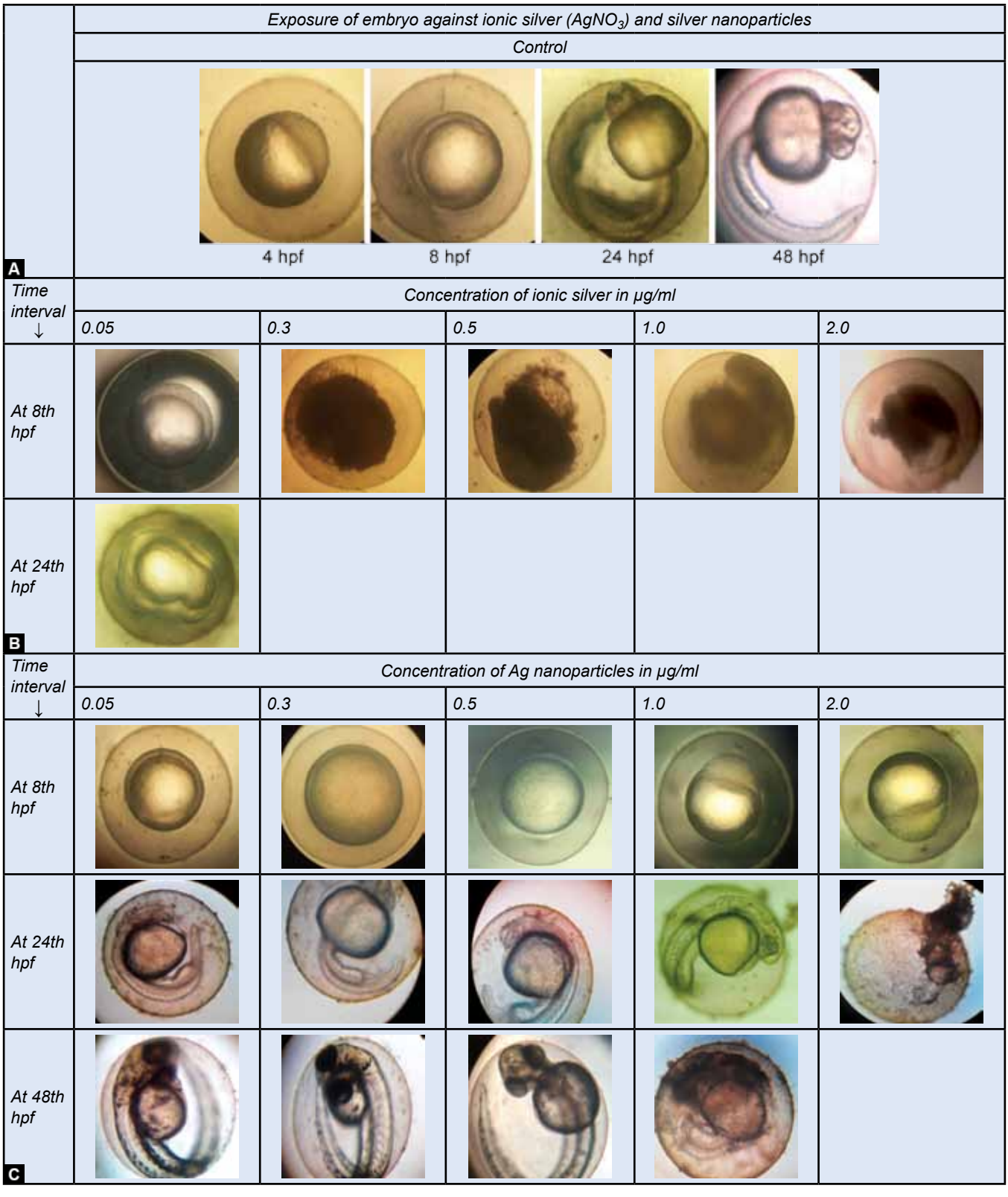
DISCUSSION

Environmental exposure to nanomaterials is inevitable as nanomaterials become part of our daily life. As a result, nanotoxicity research is gaining attention. The range of particle size used in this study was about 4 to 10 nm. Hence, the cytotoxicity profiles obtained pertain to silver nanoparticles of these sizes only. The cytotoxicity profiles of particles of greater size could be different than the one observed here.[15] In this study, zebrafish embryos were treated with AgNPs (0.05, 0.3, 0.5, 1.0 and 2.0 µg/ml) during 4 to 72 hours post fertilization (hpf). A concentration-dependent increase in mortality and hatching delay was observed in Ag-np treated embryos for 1.0 µg/ml in almost 90% cases. We find that single AgNPs passively diffuse into five different developmental stages of embryos (cleavage, early-gastrula, early-segmentation, late-segmentation, and hatching stages), indicating possible stage-independent diffusion mode. Notably, the Ag nanoparticles induce distinctive stage and dose-dependent phenotypes and nanotoxicity, upon their acute exposure to the AgNPs particularly for 2.0 µg/ml beyond 48 hpf.

In contrast, the hatching embryos seemed to sustain Ag NPs below a concentration of 1.0 µg/ml, and majority of embryos (95%) develop normally. Interestingly, early-gastrula embryos are less sensitive to the NPs than cleavage and segmentation stage embryos, and do not develop abnormally. Reasons for such stage dependent toxicity are not yet clear. Similar studies are in progress to investigate toxicity of Ag nanoparticles in rats, another vertebrate model systems.[16]

CONCLUSION

In summary, the present study demonstrates that AgNPs cause developmental embryonic toxicity beyond a concentration of 1.0 µg/ml, resulting in persistent effects on larval behavior. Thus, present studies suggest that exposure to AgNPs beyond 1.0 µg/ml could be a potential hazardous



Figs 2A to C: (A) Control embryos at different time intervals, (B and C) zebrafish embryos at 6 and 24 hpf exposed at different concentrations of ionic silver and Ag nanoparticles respectively. Blank panels indicate no survivors/dead embryos

factor for biological exposure whereas a concentration of or below 0.5 µg/ml can help prove sustainable to exploit positive effects of nanoparticle application. However, more studies of relation between AgNPs exposure, adverse effects and biological mechanisms, and studies on safety evaluation of AgNPs are needed to extrapolate these observations to human systems. Studies on such model systems thus provide valuable information on nanoparticle induced genotoxicity which could be of significance for human healthcare applications.

Table 1: Average viability of hatched embryos at 72 hpf for exposure toward ionic silver ($AgNO_3$) and Ag nanoparticles

Cong in µg/ml	*No. of embryo exposed*	*No. of live embryos exposed to ionic silver*		*No. of live embryos exposed to Ag nanoparticles*	
		Live (average)	*SD ± SE*	*Live (average)*	*SD ± SE*
0	20	17.66	2.5 ± 1.45	19.33	1.15 ± 0.66
0.1	20	11	1.15 ± 0.66	18	1.0 ± 0.57
0.3	20	1	1 ± 0.57	18	1.0 ± 0.57
0.5	20	0.33	0.70 ± 0.5	15.33	0.57 ± 0.33
1.0	20	0	0	9	1.0 ± 0.57
2.0	20	0	0	0.33	0.057 ± 0.33

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ORIGINAL ARTICLE

TG/HDL-C Ratio: A Surrogate Marker of Insulin Resistance in Patients with Metabolic Syndrome

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ABSTRACT

Insulin resistance (IR) is hallmark of metabolic syndrome. It is important to identify IR as it is the early stage before development of diabetes mellitus. The standard method to measure insulin resistance is the euglycemic clamp technique, which is laborious. Hence, a number of surrogate measures like homeostasis model assessment of insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI) and triglyceride/high density lipoprotein cholesterol (TG/HDL-C) ratio have been developed. Both of the former involve calculations, while TG/HDL ratio may be readily available for clinicians. Therefore, this study was undertaken to assess whether TG/HDL-C ratio serves as a better predictive marker of IR.

Objectives: The aim of the present study was to evaluate the triglyceride/HDL-C ratio as a surrogate marker of IR in metabolic syndrome patients.

Materials and methods: Total 110 patients were recruited in the study after obtaining informed written consent. They were divided into two groups. Group I included healthy controls (n = 50) and subjects with metabolic syndrome (MS) (n = 60) as per NCEP ATP III criteria were included in group II. Anthropometric measurements and biochemical analysis was performed in all subjects.

Results: There was statistically significant difference in anthropometric, glycemic and lipid parameters in control and study group ($p < 0.0001$). The regression model between HOMA-IR and TG/HDL-C ratio showed was positive correlation ($r = 0.29$, $p = 0.01$) while between QUICKI and TG/HDL-C ratio showed negative correlation ($r = –0.37$, $p = 0.002$).

Conclusion: We report in our study that TG/HDL-C can be adopted in routine laboratory practice as a surrogate marker for prediction of insulin resistance. So that patients with metabolic syndrome may be beneficial at an early stage.

Keywords: Insulin resistance, Triglycerides, High density lipoprotein, Metabolic syndrome, HOMA-IR, QUICKI.

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INTRODUCTION

Insulin resistance (IR) is a major finding in metabolic syndrome (MS)[1] and a contributing factor for risk of development of type 2 diabetes and cardiovascular diseases (CVD). Therefore, a reliable measure of insulin resistance is important for investigating its link with metabolic syndrome (MS).[2,3] For this, the homeostasis model assessment of insulin resistance (HOMA-IR),[4] is widely accepted method as an alternative to the glucose clamp which is laborious. HOMA-IR is comparable to the glucose clamp technique in terms of precision but not accuracy and, hence, it is possible to study large number of subjects using a single measurement of glucose and insulin in fasting state.[5] In addition to HOMA-IR, the quantitative insulin sensitivity check index (QUICKI) derived from logarithmically transformed fasting plasma glucose (FPG) and insulin levels has also proven to be a first-rate index of insulin resistance in comparison with clamp-IR.[6]

QUICKI provides a consistent and precise index of insulin sensitivity with better positive predictive power.[7,8-10] It is simply a variation of HOMA equations, as it transforms the data by taking both the logarithm and the reciprocal of the glucose-insulin product, thus slightly skewing the distribution of fasting insulin values. QUICKI has been seen to have a significantly better linear correlation with glucose clamp determinations of insulin sensitivity than minimal-model estimates, especially in obese and diabetic subjects.[8] QUICKI should not be considered, as a new model rather simply logs as HOMA-IR, which explains the near perfect correlation with HOMA.

Another alternative proposed for the identification of IR is determination of fasting triglycerides (TG) to HDL-C ratio and glucose.[11] Since elevated triglyceride is one of the NCEP ATP III criteria for MS, hypertriglyceridemic state which may accompany either normal or impaired fasting glucose should promote screening for IR.[12,13]

Plasma TG and HDL-C concentrations are independently associated with insulin sensitivity and a ratio of TG/HDL-C concentration is significantly related to a direct measure of insulin-mediated glucose disposal.[14,15]

Recently, it is proposed that triglycerides/high density lipoproteins (HDL) cholesterol ratio is one of the indices to evaluate the atherogenic state due to the association between dyslipidemia and IR. IR significantly impacts lipoprotein

metabolism and is associated with increased in TG levels and depressed HDL levels.[16,17]

The purpose of this study was to compare the existing model of HOMA-IR and QUICKI with TG/HDL-C ratio for assessment of insulin resistance in MS patients.

MATERIALS AND METHODS

This was an observational prospective study approved by Institutional Ethics Review Committee. A total of 110 subjects between age group of 35 and 65 years were recruited for the study after obtaining their informed consent. As occurrence of the disease (MS and DM) is common, the selected sample size will be representative of population and sample size was approved by institutional biostatistician. The subjects were divided into two groups. Group I included healthy controls and Group II included subjects with metabolic syndrome. All the subjects were matched for age, gender and were excluded for chronic diseases of kidney and liver as well as for cancer and diabetes mellitus. The diagnosis of metabolic syndrome was based on NCEP ATP III criteria. Measurements of height and weight were done with the subjects standing, without shoes and with light clothing. Body mass index (BMI) was calculated as weight in kg divided by height in meter squared. WC was measured at the level of the umbilicus with a tape in centimeter scale.

Venous blood samples were obtained from the antecubital vein under conditions of 12 hours of fasting. Fasting plasma glucose, TG, total and HDL cholesterol levels were measured by enzymatic techniques on fully automated analyzer. LDL and VLDL values were obtained by using Friedewald's formula.[18] Fasting plasma insulin was measured by ELISA technique. Insulin resistance was determined by means of HOMA-IR using the following formula:

HOMA-IR = fasting insulin (μIU/ml) × fasting glucose (mg/dl)/405. QUICKI was determined using the formula.[19]

$$QUICKI = 1/[\log (\text{insulin } \mu U/ml) + \log (\text{glucose mg/dl})]$$

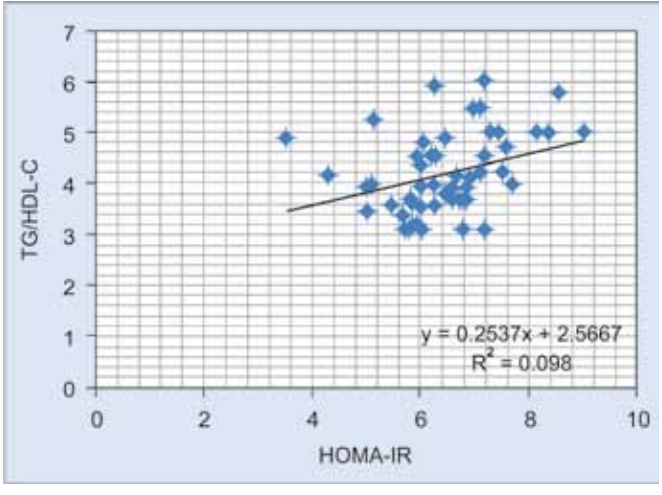
The TG/HDL-C ratio was calculated using the formula: fasting TG (mg/dl)/HDL-cholesterol (mg/dl).

STATISTICAL ANALYSIS

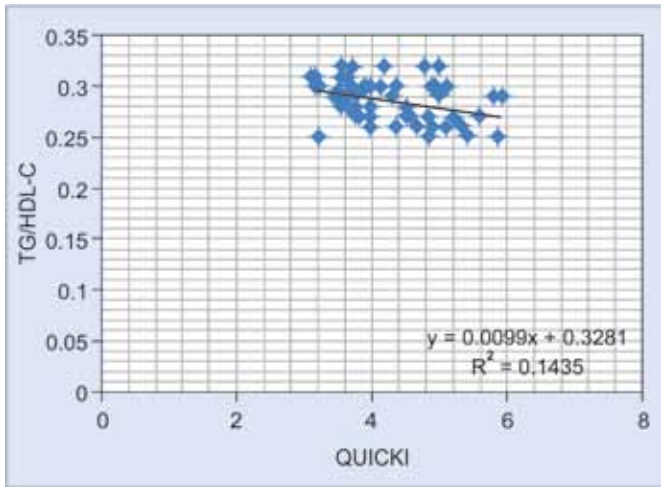
Data are presented as means ± SD, student t-test was used to compare age, BMI, W/H ratio, SBP, DBP, FPG, FPI and lipid profile levels between patients and controls. The correlation of TG/HDL-C ratio with both HOMA-IR and QUICKI was determined by Pearson correlation coefficient and simple regression model was used. Statistical Package for Social Sciences (SPSS, version 17.0) was used. p-values <0.05 were considered statistically significant.

RESULTS

Table 1 shows anthropometric and clinical characteristics of control and study group. There were significant differences in the values of BMI, waist circumference, systolic blood pressure, diastolic blood pressure in control and study group ($p < 0.0001$). The difference in fasting plasma glucose and LDL cholesterol was also statistically significant in control and study group ($p < 0.0001$). The HOMA-IR was elevated and QUICKI was decreased in metabolic syndrome group compared to controls. The regression model between HOMA-IR and TG/HDL-C ratio showed was positive correlation ($r = 0.29$, $p = 0.01$) while between QUICKI and TG/HDL-C ratio showed negative correlation ($r = –0.37$, $p = 0.002$), refer to Graphs 1 and 2 respectively. The mean high density lipoprotein-cholesterol (HDL-C) level was significantly lower in group II ($p < 0.0001$).



Graph 1: Correlation between TG/HDL-C ratio and HOMA-IR (r = 0.29)



Graph 2: Correlation between TG/HDL-C ratio and QUICKI (r = –0.37)

Table 1: Descriptive and comparative statistics for different groups by student t-test

Parameters	*Group I (control)* (n = 50)	*Group II* (metabolic syndrome) (n = 60)	**p-value*
BP (systolic) mm/Hg	115 ± 6.98	134 ± 2.86	0.0001
BP (diastolic) mm/Hg	76.7 ± 4.98	96 ± 0.60	0.0001
BMI in kg/m^2	23.3 ± 1.39	29.6 ± 0.53	0.0001
W/H ratio	0.802 ± 0.03	0.99 ± 0.01	0.0001
Fasting plasma glucose mg/dl	88.1 ± 5.06	113. ± 1.19	0.0001
Fasting plasma insulin µIU/ml	10.2 ± 3.2	22 ± 0.11	0.0001
HOMA-IR	2.23 ± 0.69	6.14 ± 0.09	0.0001
QUICKI	0.34 ± 0.03	0.29 ± 0.02	0.0001
Cholesterol mg/dl	146.3 ± 10.59	174 ± 15	0.0001
Triglycerides (TG) mg/dl	110 ± 17.2	189 ± 7.5	0.0001
High density lipoprotein cholesterol (HDL-C) mg/dl	44.5 ± 3.58	40 ± 0.6	0.0001
Very low density lipoproteins mg/dl	21.59 ± 2.72	36 ± 2.1	0.0001
Low density lipoproteins mg/dl	83.92 ± 14.2	103 ± 22.2	0.0001
TG/HDL-C ratio	2.4653 ± 0.2094	4.554 ± 0.152	0.0001

Data are expressed as mean ± SD; *Student's t-test for independent samples

DISCUSSION

In our study, we have used HOMA-IR and QUICKI, the widely accepted model for assessment of IR and compared with TG/HDL-C as marker of IR.

The TG/HDL-C ratio was calculated in metabolic syndrome patients and control group and we found that TG/HDL-C ratio was higher in the study group. There was significant positive correlation between HOMA-IR and TG/HDL-C ratio ($r = 0.29$; $p < 0.05$) in the study group. The regression model showed R2 value of 0.09, indicating 9.0% variation in the HOMA-IR due to TG/HDL-C ratio, refer to Graph 1.

Our results are in accordance with Salazar et al[20] Morato et al[21] and Brehm et al[22] who demonstrated positive correlation between TG/HDL-C ratio and insulin resistance, confirming that TG/HDL-C ratio predicts insulin resistance in metabolic syndrome. But, an independent study by Knight et al[23] stated that triglyceride/high-density lipoprotein cholesterol ratio fails to predict insulin resistance in African-American women.

The regression model of TG/HDL-C and QUICKI showed strong negative correlation ($r = –0.37$; $p < 0.005$). The R^2 value showed by regression model is 0.14 showing 14% variation in QUICKI due to TG/HDL-C ratio, refer to Graph 2. These finding are similar to the finding of Brehm et al who found that QUICKI indicated severe insulin resistance in individuals with impaired glucose tolerance.[22]

Thus, the regression model indicates that TG/HDL-C ratio is a significant variable of insulin resistance in metabolic syndrome patients. Most of the studies have compared HOMA-IR with either TG alone or with TG/HDL-C ratio.[21,24] But, combined model of TG/HDL-C with HOMA-IR and QUICKI has been reported in very few studies. TG/HDL-C ratio is an economic and it is easy to calculate[25] and a good predictor of LDL size. This ratio offers the most practical approach to identify insulin resistance. Moreover, it is associated with a higher risk for cardiovascular diseases than just serum TG concentrations in a population including 8 to 14% diabetic patients.[26] These data suggest that the TG/HDL-C ratio may serve as a surrogate marker of IR. It is easy to determine and links insulin resistance and cardiovascular risk in nondiabetic individuals.

This study is limited by the fact that HOMA-IR index and QUICKI was not correlated with insulin sensitivity by the gold standard method, such as euglycemic clamp.[14]

CONCLUSION

This study demonstrates that TG/HDL-C ratio positively correlates with insulin resistance in metabolic syndrome patients. Therefore, we propose that TG/HDL-C ratio serves as an easily available and economic laboratory marker for the busy clinicians to identifying insulin resistance in metabolic syndrome patients.

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AUTHORS' CONTRIBUTIONS

Ms Parineeta Samant performed the biochemical assays and assisted in writing the paper. Dr Padma Chavan analyzed the results critically and wrote the paper. Dr Sandeep Rai gave valuable inputs in the manuscript. All authors read and approved the final manuscript.

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REVIEW ARTICLE

Diagnosis and Effective Management of Preterm Labor

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ABSTRACT

Despite extensive research, we are still unable to diagnose, prevent and treat preterm labor. Monitoring efficacy of interventions that would allow this is largely biased by the inability to accurately identify true labor with the currently used crude technology. Progestin supplementation appears to be a promising approach to both preventing initiation of preterm labor and treating it once it is already established, given progesterone's role in maintaining pregnancy as well as support from basic and clinical research. However, the questions on mechanisms of action, optimal progestin formulation, dose, route and timing of administration remain unanswered. We have established and reported noninvasive means to accurately monitor cervical ripening, by measuring collagen light-induced fluorescence (LIF) and myometrial contractility, by measuring uterine electromyography (EMG). By accurately assessing the two components of parturition, cervical LIF and uterine EMG can help to identify effective prevention strategies and treatment of preterm labor.

Keywords: Labor and delivery, Uterine electromyography, Cervical light-induced fluorescence, Progesterone, 17 alpha-hydroxyprogesterone acetate.

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INTRODUCTION

Preterm birth remains the biggest unsolved obstetrical problem. As much as 70% of perinatal mortality is attributed to prematurity, and many of the surviving preterm infants suffer serious lifelong morbidity, including cerebral palsy, blindness, hearing loss, learning disabilities and other chronic conditions.[1-3] In spite of extensive research and a variety of interventions, the incidence of preterm birth has not declined.[4] Presently, there are about 15 million babies born prematurely in the world among a total of about 150 million births per year.[5] In Europe, the preterm birth rate varies from about 5 to 11%, with relatively lower preterm birth rates in Finland, the Baltic countries, France and Sweden, and higher rates in Austria and Germany. Births occurring before 32 weeks of gestational age, when the risk of death and handicap is especially increased, account for about 1% of all births.[2,6,7] Even more disturbingly, in the United States the incidence of preterm birth has been consistently rising.[8] A more than 20% increase in the preterm birth rate was observed between 1990 and 2006.[9] Births after 32 weeks accounted for most of this increase, but births before 32 weeks also increased.[9] There has been a slight decline in the incidence of prematurity recently, but this has been primarily among late preterm births.[10]

Most common interventions recommended to prevent preterm birth, such as bed rest, tocolytics, antibiotics and cervical cerclage have been proven to have little or no benefit.[8] Once preterm labor is established, the goal of treatment is merely to delay delivery in order to allow for the transfer of the pregnant patient to the most appropriate hospital and for administration of corticosteroids.[11] None of the currently available treatments for preterm labor can prolong pregnancy sufficiently to allow further intrauterine growth and maturation of the fetus.[12] There is experimental support from animal and *in vitro* studies, and also empirical evidence from large randomized placebo-controlled clinical trials, that treatment with progestins may reduce the risk of preterm birth in both high-risk asymptomatic patients and in those presenting with signs and symptoms of preterm labor. Progestins are a group of steroid hormones that include natural progesterone and its analogs, such as 17 alpha hidroxyprogesterone caproate (17P) and medroxyprogesterone acetate (MPA).

Research of progestin and other potential treatments for preterm labor is largely hindered by the inability to reliably distinguish patients who are going to deliver preterm from those who are not. The analyses of effects of treatments are largely confounded by inclusion of patients who would not deliver preterm regardless of intervention. The first step in finding an effective prevention and treatment for preterm labor is, therefore, finding a method that will allow targeting

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the treatment only to patients who would, if not treated, really deliver preterm.

Model of Parturition

Parturition, both at term and preterm, is a complex process involving ripening of the uterine cervix and activation of the myometrium. Understanding and accurate assessment of these two components is the key to reliably predict and effectively treat preterm labor.

Cervix

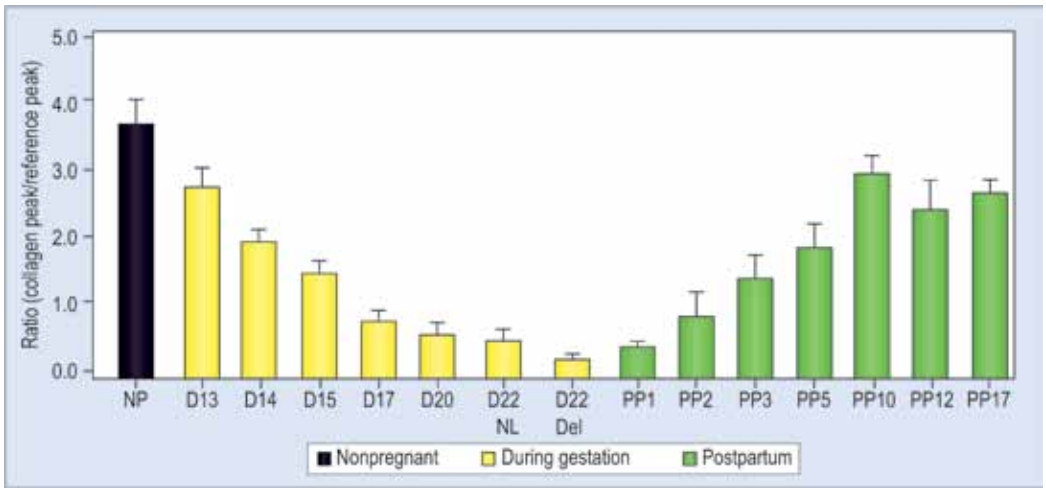
The term 'cervical ripening' summarizes many biochemical and functional changes that result in the softening, effacement, and dilatation of the cervix, eventually allowing the delivery of the fetus. During this progressive event, the connective tissue in the cervix, consisting predominantly of collagen, is degraded and rearranged.[13] Cervical ripening does not depend on uterine contractions and is similar to an inflammatory reaction. It involves the infiltration of polymorphonuclear cells and a release of degradative enzymes—metalloproteinases, resulting in a decrease of collagen concentration in the tissue.[14] The changes in collagen content in the cervix, and consequently the degree of cervical ripening, can be assessed noninvasively by measuring the light-induced fluorescence (LIF) of the non-soluble collagen (Graph 1).[15]

Myometrium

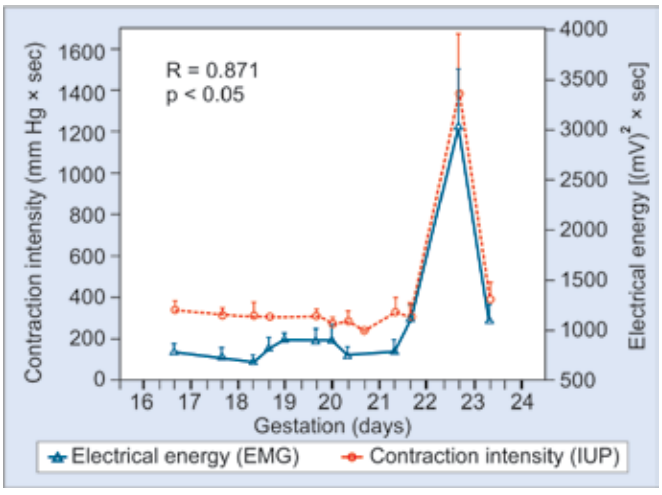
Several events in the myometrium precede labor. Excitability of cells increases due to changes in transduction mechanisms and synthesis of various proteins, including ion channels and receptors for uterotonins.[16-18] At the same time, systems that inhibit myometrial activity, such as nitric the oxide system, are downregulated, leading to withdrawal of uterine relaxation.[15] Electrical coupling between myometrial cells also increases, and an electrical syncytium allowing the propagation of action potentials from cell to cell is formed.[19,20] These changes are required for effective contractions that result in the delivery (expulsion) of the fetus. The transition from the nonlabor to the labor state of the myometrium can be identified by monitoring the uterine electromyographic (EMG) activity from the abdominal surface noninvasively.[15,21,22] An increase in uterine EMG activity corresponds to the increase of uterine contractility immediately preceding delivery in rats (Graph 2). Changes in certain EMG parameters, such as power spectrum (PS) peak frequency and amplitude, and propagation velocity of uterine electrical signals, also indicate the onset of true labor at term and preterm in humans (Graph 3B).[21,23-25]

Timeline of Events

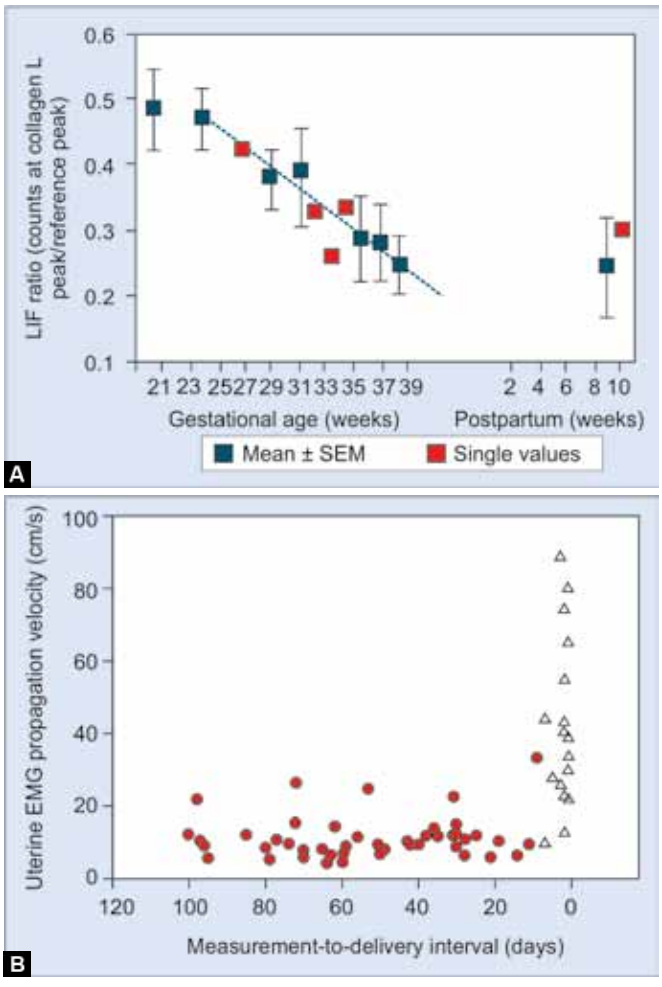
The two components of parturition, i.e. cervical ripening and activation of the myometrium, take place in a different time frame. Studies of cervical LIF showed that the process of softening and shortening of the cervix starts in mid-pregnancy, or even sooner (Graph 3A).[26] Although cervical LIF and cervical length have not been directly compared yet, this seems to be in accordance with transvaginal ultrasound studies which showed that the cervix gradually shortens throughout gestation.[27] The myometrial activation, in contrast, is a more acute event, occurring relatively close to delivery. In rats, the uterine EMG activity increases not more than 24 hours before delivery (*see* Graph 2).[28] Similarly, in humans, the increases of EMG PS peak frequency and propagation velocity, which accurately identify myometrial preparedness for labor, do not typically occur more than seven days from delivery preterm and generally even later at term (*see* Graph 3B).[24,25]



Graph 1: Gradual changes in rat cervix during cervical ripening assessed by measuring the light-induced fluorescence (LIF) of collagen shown are LIF measurements as ratios of the collagen peak *vs* the reference peak values in nonpregnant rats, during different times of pregnancy (day 13 to day 22) and postpartum (day 1 to day 17)



Graph 2: Acute changes in myometrial activity preceding delivery in rats. Note the excellent correlation between contraction intensity measured by intrauterine pressure catheter (IUP) and energy of uterine EMG signals



Graphs 3A and B: (A) Cervical LIF ratio throughout human pregnancy and postpartum, (B) uterine EMG propagation velocity increases immediately prior to delivery (Δ: delivery ≤ 7 days from the measurement; •: delivery > 7 days from the measurement)

In conclusion, parturition is composed of cervical ripening and myometrial contractility. The changes in the cervix and the muscle leading to delivery of the fetus take place in a different time frame. Cervical ripening is a slower process which is initiated already in mid pregnancy, whereas the myometrium becomes activated acutely, just prior to the delivery.

Diagnosis of Preterm Labor

The diagnosis of preterm labor today still often relies on presence of contractions assessed by tocodynamometry (TOCO) and cervical change assessed by digital cervical examination. However, contractions occur commonly in normal pregnancy, and their detection through maternal perception and/or TOCO has a low sensitivity and positive predictive value for preterm delivery.[29,30] Moreover, digital cervical examination suffers from large variations among examiners, and its prognostic values have also been shown to be low.[31,32]

There is substantial evidence that measuring the cervical length by transvaginal ultrasound and testing for fetal fibronectin in cervicovaginal fluid can help to identify patients at particularly high-risk for preterm delivery.[33-35] Cervical length is inversely related to the rate of preterm delivery in both patients presenting with symptoms of preterm labor and in asymptomatic pregnant women.[33,34,36,37] Fetal fibronectin is an extracellular matrix glycoprotein produced by amniocytes and by cytotrophoblast, that normally resides at the decidual-chorionic interface.[35] Its presence in the cervicovaginal fluid indicates decidual activation. However, the value of these two tests lies mostly in their high negative predictive values (NPV), while their positive predictive values (PPV) are lower and they do not identify patients who are really going to deliver preterm reliably.[38]

None of the currently used methods can, therefore, distinguish between true and false preterm labor reliably. This results in unnecessary treatments, missed opportunities to improve outcome of premature neonates, and also our inability to analyze the effects of treatments which largely hamper the development of more beneficial therapeutic approaches.

Role of Light-induced Fluorescence of Collagen

Disruption of collagen in the cervical extracellular matrix occurs prior to delivery at term and preterm.[13] The methods currently available to clinicians to assess these changes in the cervix have several major drawbacks. Digital cervical examination is subjective, and not accurate in predicting preterm delivery.[31,32] Measurement of cervical length by transvaginal ultrasound is a more reproducible method, and has a high NPV. It therefore reliably identifies patients in whom the probability of preterm delivery is very low,

but a short cervix does not necessary mean that the patient is really going to deliver preterm.[33,34] As mentioned previously, we have shown that cervical collagen content can be monitored noninvasively by measuring LIF of collagen.[39] This methodology allows an objective assessment of the change in cervical structure, and can detect the change in the composition of the cervix, regardless of its length. It is, therefore, a more accurate method to diagnose cervical ripening. It can potentially detect pregnant women at risk of preterm birth well before changes in cervical length occur.

Role of Uterine Electromyography

Previous studies have established that the electrical activity of the myometrium is responsible for myometrial contractions (Fig. 1).[40,41] Extensive studies have been done to monitor uterine contractility using the electrical activity measured from electrodes placed directly on the uterus.[42,43] More recent studies published by our group and by others indicate that uterine EMG can be monitored non-invasively from the abdominal surface (Fig. 2).[15,21,22,44,45]

Measuring uterine EMG activity has similar effectiveness of simple detection of uterine contractions as does TOCO, and even as compared to intrauterine pressure catheter.[46-49] In addition, many studies have shown that different uterine EMG parameters can indicate myometrial properties that distinguish physiological preterm contractions from true preterm labor, which is something that the other contraction-monitoring devices cannot do.[24,44,50,51]

Of all of the possible EMG diagnostic variables, 'timing related' EMG parameters seem to have the least predictive value. We recently analyzed the duration of uterine EMG 'bursts', the interburst interval duration (which is inversely proportional to the frequency of the bursts) and the standard deviation of burst and interburst interval duration in patients admitted with the diagnosis of preterm labor at <34 weeks. None of these parameters differed significantly between the group of patients who delivered within 7 days and those who did not.[25] This is not in accordance with some studies, which found that the standard deviation of burst duration was smaller, and the frequency of burst was higher in labor patients.[50,52] We did, however, confirm the findings of Leman et al and Buhimschi et al, who observed no differences in burst duration between preterm labor patients and women with preterm contractions that did not deliver preterm.[44,50] Burst duration and frequency of bursts are the electrical equivalent of the duration and frequency of contractions, and these, not coincidentally, are the only properties of contractions that can be evaluated by TOCO. Thus, their poor predictive values are not surprising, since monitoring uterine activity with TOCO is not helpful in identifying patients in preterm labor.[29,49]

Another type of EMG parameters can be categorized as 'amplitude related'. Such parameters may represent the uterine EMG signal power or alternatively, the EMG signal energy. Buhimschi et al demonstrated that an increase in PS

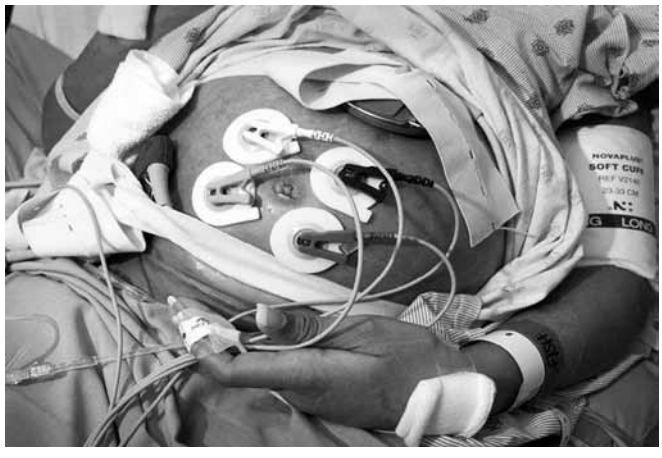


Fig. 2: Electrode placement on the abdominal surface of the patient for performing uterine EMG measurement, to diagnose preterm labor or to monitor the efficacy of treatment for preterm labor

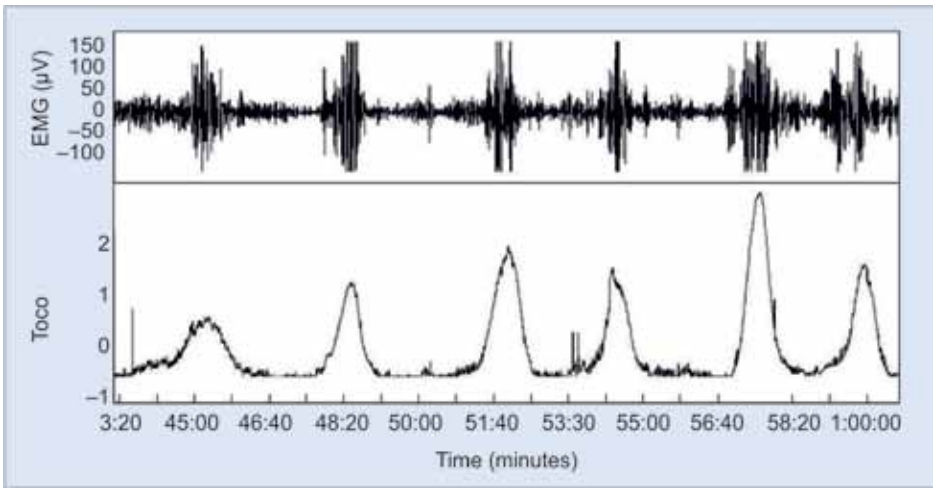


Fig. 1: Electrical activity of the myometrium (EMG activity—top trace) is responsible for uterine contractions. Note the excellent temporal correspondence between EMG and mechanical contractile events (measured by IUPC—bottom trace)

peak amplitude precedes delivery.[50] Other studies did not confirm these findings.[23,24,52] In the previously mentioned study on preterm labor patients, neither power spectrum (PS) peak amplitude nor PS median amplitude were significantly higher in patients who delivered within 7 days compared to those who did not. It has been suggested that the major limitation of 'amplitude related' EMG parameters is the fact that attenuation of myometrial signals occurs more for some patients and less for others, depending on a variance in subcutaneous tissues, and a variance in conductivity at the skin-electrode interface. These limitations make the 'amplitude related' EMG parameters interesting, but perhaps less reliable, in the prediction of preterm labor.

The third group of EMG parameters can be defined as 'frequency related' parameters and it includes PS median and peak frequency. Median frequency, although usually the most important parameter in the analysis of striated muscle EMG,[53-55] is rarely reported to be useful in the uterine EMG literature.[45] The reason for that is probably the difference in the PS of the signals from the uterine and striated muscle cells. The PS of a striated muscle covers a broad frequency range (20-400 Hz), with a more or less bell-shaped distribution of signal energy. Thus, for striated muscle, the median frequency is a most useful parameter in the analysis of these signals. On the other hand, uterine EMG signals are filtered in order to exclude most components of motion, respiration and cardiac signals, which yields a narrow 'uterine-specific' band of 0.34 to 1.00 Hz. In this narrow frequency band produced by the uterus, the location of the power peak differs from one recording to another, and there are often competing 'lesser' power-spectral peaks, not generally of consequence in the broad power-spectra of striated muscle. This suggests that the type of narrow-band power distribution found in the uterine-specific range of frequencies may render using the median frequency a less useful parameter for characterizing the uterine electrical signals. Verdenik et al have, however, reported that as pregnancy approaches term, the median frequency of the uterine electrical activity becomes lower.[45] It is not clear why this should be so, since other literature supports shifts to higher frequencies as a transition to labor occurs.[42] Furthermore, shifts to lower median frequency in the electrical PS of muscle are generally attributed to muscle fatigue.[54] A possible explanation for this is that the median PS frequency for the whole 30 minutes EMG recording, and not for each burst separately, was analyzed in that study. It may be that including non-uterine related electrical information (from the large portions of the recordings 'in-between' bursts) contributed somehow to this result.

In contrast, PS peak frequency has been one of the most predictive EMG parameters in both human and animal studies.[22,24,52] Shifts to higher uterine electrical signal peak frequencies occur during transition from a nonlabor state to both term and preterm labor states, and can be reliably assessed by noninvasive transabdominal uterine EMG measurement.[24,44] PS peak frequency also increases as the measurement-to-delivery interval decreases.[24] The best predictive values of PS peak frequency have been identified at different measurement-to-delivery intervals by different authors.[24,44] Generally, an increase in PS peak frequency occurs within approximately 24 hours from delivery at term, and before that (within several days from delivery) at preterm gestations.[24,25]

We have recently explored a new EMG parameter: the propagation velocity (PV) of uterine EMG signals. It has been shown *in vitro* that the PV of electrical events in the myometrium is increased at delivery when gap junctions are increased.[56,57] We demonstrated that PV can be assessed from the noninvasive uterine EMG recording *in vivo* by estimating the time interval between EMG signal arrivals at adjacent electrode pairs.[25] We have also shown that PV may predict preterm delivery more reliably than any other EMG parameter investigated so far.[25]

Both EMG PV and PS peak frequency more accurately identify true preterm labor than today's clinical methods (Graph 4).[25] By combining the PV and PS peak frequency, we constructed a model that predicted spontaneous preterm birth with and area under the receiver-operating-characteristics curve of 0.96 (Table 1). This makes this methodology extremely valuable in everyday clinical practice. When uterine EMG is measured in patients presenting with signs and symptoms of preterm labor and the combination (rescaled sum) of PV and PS peak frequency exceeds the cut-off value of 84.48, this predicts delivery within 7 days

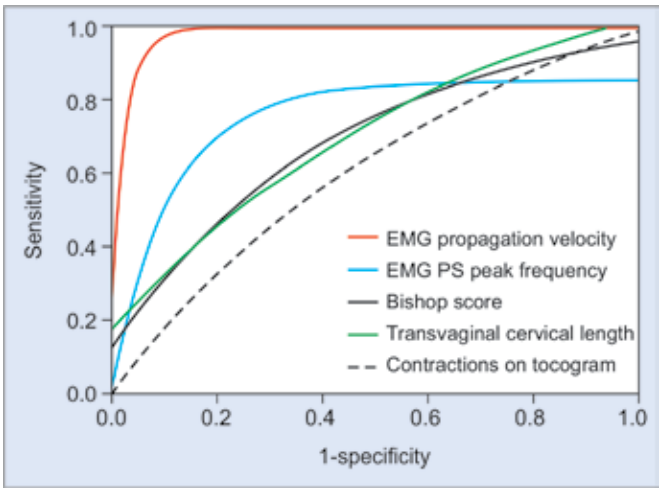
Table 1: Predictive measures of uterine EMG parameters [power spectrum (PS) peak frequency, PS peak amplitude and propagation velocity] compared to current methods to predict preterm delivery

Method	*AUC*	*Best cut-off*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
EMG (PV + PS peak frequency)	0.96	84.48	70	100	100	90
Bishop score	0.72	10	18	100	100	81
Transvaginal cervical length	0.67	0.7 cm	14	98	50	90
Contractions on TOCO	0.54	N/A	35	72	27	79

with a 100% certainty according to our data (PPV = 100% in 88 patients). EMG does, therefore, identify patients who really benefit from early institution of tocolytic therapy, transport to a hospital with facilities for neonatal intensive care, and administration of steroids. At the same time, this methodology also identifies patients in false preterm labor who are not going to deliver within the next 7 days. It can, therefore, help to avoid substantial economic costs associated with unnecessary hospitalization, the maternal risks associated with tocolytics, and the potential fetal risks associated with steroids. In the case of low PV + PS peak frequency values, it therefore stands to reason that it would be safe not to admit, treat, or transfer the patient, regardless of the presence of contractions on TOCO, and regardless of digital cervical examination and transvaginal cervical length results, since all of the changes in the myometrium required for labor are not yet fully established. Other than being extremely important clinically, a methodology to accurately diagnose preterm labor would also be important in the research of new and potentially better treatments for preterm labor.

Importance of Diagnostic Tests for Monitoring Efficacy of Treatment for Preterm Labor

The current standard treatment for preterm labor, i.e. tocolytics, was not shown to have any clear effect on perinatal mortality or on any measure of neonatal morbidity related to prematurity.[12] Research of new and potentially more effective treatments for preterm labor is, therefore, extremely important. Development of such treatments, however, depends largely on the tests used to diagnose labor. Current methods do not allow to distinguish patients in true preterm labor, who would deliver preterm if not treated, from those in 'false' preterm labor, who present with signs and symptoms of labor, but would not deliver preterm regardless of treatment. The overall PPV of currently used methods to predict preterm delivery is only about 50%, as mentioned above.[58] This inevitably leads to inclusion of patients in 'false' labor into studies of effectiveness of treatments.



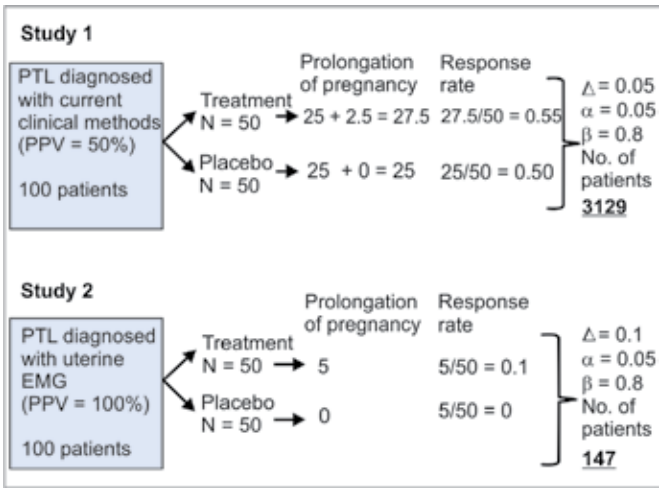
Graph 4: Comparison of receiver operating characteristic curves for EMG parameters [power spectrum (PS) peak frequency and propagation velocity] *vs* currently used methods to predict preterm delivery

A method with higher diagnostic accuracy, such as uterine EMG, would allow the performance of clinical trials on just patients who are really in true preterm labor. It has been shown, that using the EMG of the uterus we are be able to predict preterm delivery very accurately (with a PPV of 100% based on our data) (Graph 4 and Table 1).[25]

The impact that this technology could have on investigation of new treatments can be illustrated by a hypothetical case of a 10% effective treatment, i.e. a treatment that would prolong pregnancy more effectively than tocolytics in 10% of patients. In order to test for the efficacy of such treatment one would need to compare a group of patients treated with the drug to a group of patients treated with a placebo. Consider the two studies presented in Flow Chart 1. In the first study preterm labor would be diagnosed with currently available methods. Fifty of 100 patients diagnosed as being in preterm labor will not deliver preterm regardless of whether they will be treated or not. On the other hand, 50 of these 100 patients will deliver preterm if not treated. With equal randomization to treatment groups, 50 patients will receive the 10% effective treatment. Twenty-five of these will not be in true preterm labor, and 25 will be in true preterm labor. Around 2.5 patients in true labor will therefore not deliver preterm due to treatment. Consequently, of the 50 treated patients diagnosed clinically as being in preterm labor, 27.5 (25 who were not at risk in the first place and 2.5 at risk patients who responded to treatment) patients will not deliver preterm. The response rate will, therefore, be 55% (27.5/50 = 0.55). In the placebo group, 25 patients will not deliver preterm because they were not in true labor in the first place, thus 50% response rate (25/50 = 0.50). The difference in treated *vs* placebo group is only 0.05. Utilizing a calculator for sample size based on proportions, one would need 3129 total patients recruited to the study to find this small difference (with an alpha of 0.05 and 0.80 power).

In the second study, uterine EMG would be utilized to diagnose preterm labor. As a result, all 100 patients will theoretically be in true labor (PPV=100%). If patients are randomized equally and there is a 10% response rate, five patients (10%) in the treatment group will respond (not deliver preterm), and 0 in the placebo group. Utilizing sample size calculator based on proportions, one would need only 147 patients to find this large difference (again with an alpha of 0.05 and 0.80 power).

Flow Chart 1: The sample size calculation for two studies examining a hypothetical 10% effective treatment for preterm labor. In study 1, preterm labor is diagnosed by currently available methods. Consequently, 50% of patients included are not in true preterm labor and will not deliver preterm regardless of treatment. To demonstrate a 10% effect of treatment with an alpha of 0.05 and 0.80 power, 3,129 patients would have to be included in study 1. If preterm labor would be diagnosed by uterine electromyography (EMG) (study 2), all the patients included would be in true preterm labor (PPV = 100%). Only 147 patients would have to be included in this study to demonstrate the same efficacy with the same power



Increased effectiveness of treatment will lower the values in both studies. With a 30% effective treatment, e.g. one would need to include 339 patients in the first study, i.e. when currently available methods would be used to diagnose preterm labor, and only 42 patients when utilizing uterine EMG.[59]

This example emphasizes the importance of diagnostic accuracy of uterine EMG. It will not only allow physicians to make safer and more cost-effective clinical decisions but will also eventually lead to development of treatments for preterm labor that will improve neonatal outcome. Even if such treatment existed already, today one would need to include 3,129 patients in a study to demonstrate a 10% efficacy. On the other hand, the same rate of efficacy could be demonstrated on just 147 patients using uterine EMG to diagnose preterm labor.

Management of Preterm Labor

Prevention of preterm birth can be categorized as primary when aimed at prevention and reduction of risk factors in the general population, secondary when the aim is to identify and treat individuals with increased risk, and tertiary when treatment is initiated after preterm labor is already established. Although some interventions have been proven to be beneficial in selected population of pregnant women with certain risk factors, more than 50% of patients who deliver preterm have no apparent risk factors.[60] Therefore, the tertiary prevention of preterm birth, i.e. treatment of preterm labor, is crucial in lowering the burden of prematurity.

Current Treatment of Preterm Labor

For several decades, stopping uterine contractions, i.e. tocolysis, has been the focus of treating preterm labor. The reason for this is the incorrect assumption that uterine contractions detected by the patient or TOCO indicate the changes in the myometrium responsible for initiation of labor. Inhibition of contractions should, therefore, prevent preterm delivery and reduce neonatal mortality and morbidity. Unfortunately, however, this is not the case. Neither do clinically used methods to assess uterine contractility detect the molecular changes characteristic of myometrial activation and true labor, nor have tocolytic agents available today been shown to improve neonatal outcome.[12] Consequently, it is therefore only reasonable to use tocolytics in preterm labor patients who need to be transferred to a hospital with facilities for neonatal intensive care and in those who have not yet completed a full course of antenatal corticosteroids, since tocolytics reduce the proportion of births occurring within seven days from the beginning of treatment but do not improve outcomes *per se*.[12]

Mechanisms of Action of Tocolytics

Several pharmacologic agents are currently used to achieve tocolysis: beta-adrenergic agonists (e.g. terbutaline); magnesium sulfate; nitric oxide donors (e.g. nitroglycerin), calcium channel blockers (e.g. nifedipine); cyclooxygenase inhibitors (e.g. indomethacin), and oxytocin receptor antagonists (e.g. atosiban). These agents cause uterine relaxation by several mechanisms: Beta-adrenergic agonists increase the levels of intracellular cyclic adenosine monophosphate (cAMP), which inactivates myosin light-chain kinase and consequently inhibit contractility.[61] However, the ability to generate and react to cAMP decreases when the myometrium is preparing for labor. Magnesium sulfate hyperpolarizes the plasma membrane, decreases the intracellular concentration of calcium, and inhibits myosin light-chain kinase by competing with intracellular calcium.[62,63] Nitric oxide donors accomplish muscle relaxation via an increased production of cyclic guanosine monophosphate (cGMP) that also inactivates myosin light-chain kinase.[64] Nevertheless, like cAMP, the ability to generate and react to cGMP also decreases during labor. Calcium channel blockers inhibit the influx of calcium ions through the plasma membrane and the release of intracellular calcium from the sacroplasmatic reticulum, leading to a decrease in calcium-mediated activity of myosin light-chain kinase.[65] Cyclooxygenase inhibitors achieve tocolysis by suppression of prostaglandin synthesis.

Prostaglandins increase the formation of myometrial gap junctions and increase the intracellular concentration of calcium by raising its transmembrane influx and its release from sarcoplasmatic reticulum.[66] And finally, oxytocin receptor antagonists compete with oxytocin for binding to its receptors. They consequently reduce the oxytocin-mediated conversion of phosphatidylinositol triphosphate to inositol triphosphate which causes the release of calcium from the sarcoplasmatic reticulum.[67]

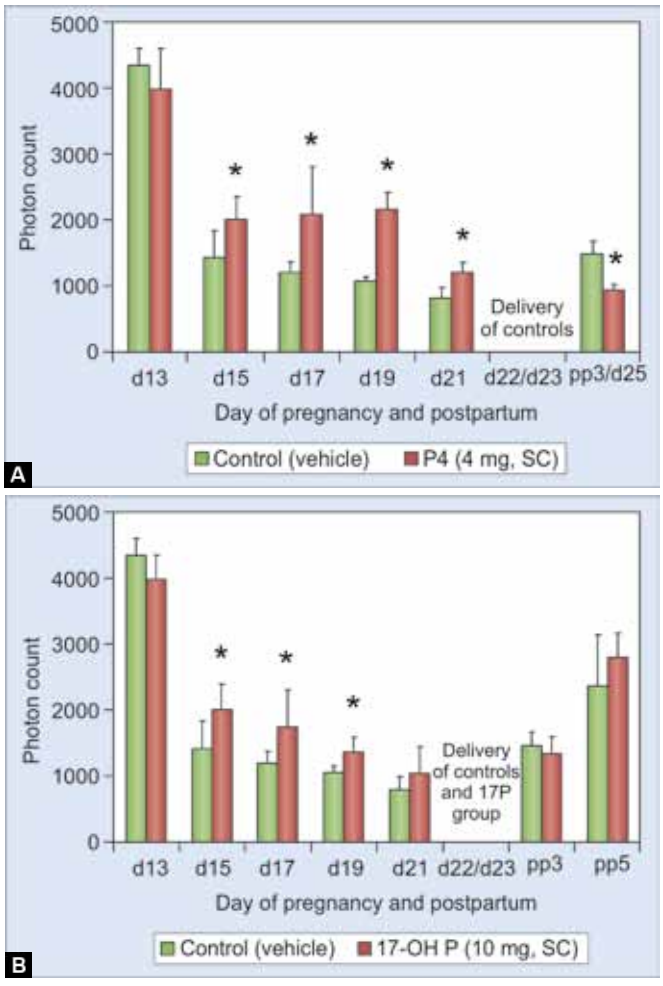
To summarize, currently used tocolytics inhibit myometrial contractility through altering the intracellular transduction pathways responsible for cell contraction, inhibiting the synthesis of myometrial stimulants, or blocking the actions of myometrial stimulants. None of them, however, can reverse the processes leading to activation of the myometrium during labor at term or preterm.

Use of Progestins to prevent Preterm Birth

Progesterone has been known to be important in maintaining pregnancy for more than 80 years, since the classic work of Corner, Allen and Csapo.[68,69] A large body of experimental data available today demonstrates that progesterone exerts overall control on both cervical ripening and myometrial contractility. Supplementation of progesterone or its analogs seems, therefore, a very promising strategy for prevention of preterm birth.

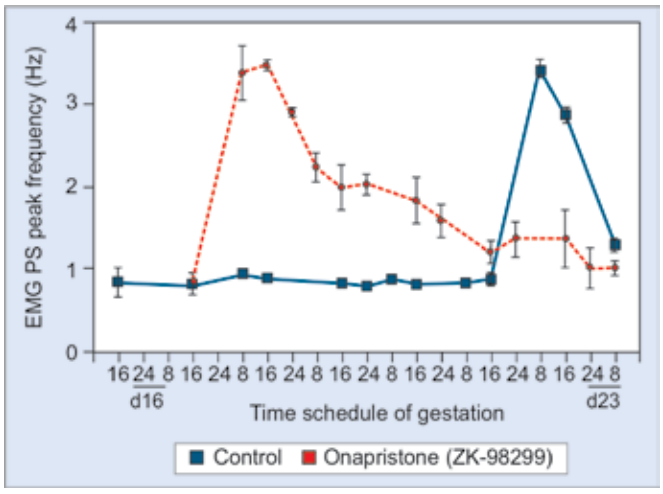
Antiprogestins induce ripening of the uterine cervix.[70] Therefore, the cascade of events leading to cervical ripening seems to be controlled at least in part by progesterone. In the cervix, progesterone modulates the expression of various genes, including those involved in regulation of epithelial and endothelial permeability and metabolism of components of the extracellular matrix.[71] Progesterone also inhibits the ripening process by suppressing the production of proinflammatory cytokines and consequently reducing prostaglandins in the cervix.[72] In a recent study, we used cervical LIF to study the effects of progesterone treatment on cervical ripening in rats. Subcutaneous (SC) and transdermal administration of progesterone significantly delayed cervical collagen degradation but did not completely suppress ripening.[73] SC administration of 17P also delayed cervical ripening, although less effectively than did progesterone (Graphs 5A and B). This is in accordance with the results of some clinical trials (discussed below) that observed an attenuation of cervical shortening measured by ultrasound with intramuscular (IM) 17P and vaginal progesterone treatment.[27,74,75]

Progesterone also inhibits myometrial activity by several mechanisms. It suppresses a number of genes that are essential for effective uterine contractions, including genes

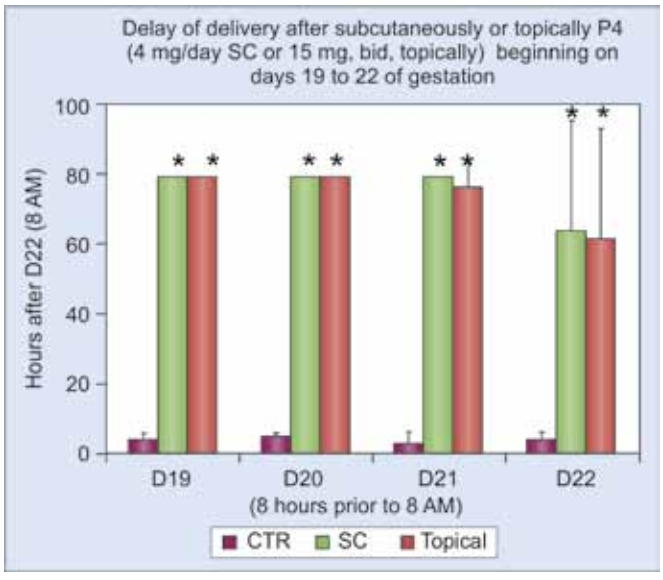


Graphs 5A and B: Means ± SD of cervical light-induced fluorescence (LIF) obtained *in vivo* from pregnant rats at different days of pregnancy and postpartum treated with progesterone (P4), 17P or vehicle. (A) Daily treatment with vehicle (controls) or P4 (4 mg, SC). Note that delivery is inhibited in the treatment group, (B) treatment daily with vehicle (controls) or 17P (10 mg, SC). Note that significant differences are only observed until day 19 of gestation

for the gap junction protein connexin 43, calcium channels and oxytocin receptors, etc.[15] It also upregulates the relaxation mechanisms, such as the generation and action of cAMP and cGMP.[15] In addition, progesterone acts by functionally opposing estrogen, which increases myometrial contractility.[15] Treatment with onapristone (ZK-98299), a pure antiprogestin, induces preterm delivery and increases the EMG activity in rats (Graph 6).[28] Recently, we have examined whether progesterone inhibits birth at term in rats. We administered micronized progesterone topically and SC daily beginning from day 19, day 20, or day 21 of gestation and 8 hours before normal delivery on day 22. All topical or SC treatments prevented birth up to 80 hours beyond the normal time of delivery observed in control, vehicle treated, rats (Graph 7). The significance of these observations is that progesterone can prevent birth when given after the decline in progesterone levels in the blood and even after the cervix



Graph 6: Treatment with onapristone (ZK-98299) on day 16 of pregnancy induces preterm delivery and an increase the EMG PS peak frequency in rats



Graph 7: The delay in delivery in pregnant rats treated daily with parenteral (4 mg subcutaneous injection, SC, of micronized progesterone) or topical progesterone (2 times daily, 15 mg micronized progesterone in fish oil) for 3 days (days 19-21), 2 days (days 20-21), 1 day (day 21) or 8 hours before normal delivery (day 22) on day 22 at 8 AM of gestation compared to controls (CTR). Note that all progesterone treatments substantially delay delivery, even when progesterone is given 8 hours prior to normally delivery. All animals treated with progesterone were sacrificed at 80 hours following 8 AM on day 22 of gestation. This study illustrates that progesterone can prevent delivery if given prior to the end of gestation at times when the cervix is already soft and prepared for delivery

is already ripened and prepared for delivery.[76] To further examine, the effects of progesterone on uterine.

Electromyography (EMG) activity we used anesthetized rats at term and sometimes animals treated with antiprogestins which were delivering preterm. We placed electrodes directly on the uterus and recorded EMG activity by conventional recording equipment as we have done previously. We recorded EMG activity over several hours and remarkably these animals delivered fetuses, although sometimes the fetuses were not completely passed through the cervix, probably because the animals were under anesthesia and did not contract the abdominal muscles or push during delivery. We noted that the EMG activity was extremely high (Fig. 3) and very similar what we had observed previously in animals fitted with internal EMG devices to measure activity without anesthesia.[28] Animals treated with progesterone 1 to 2 days prior to normal delivery had very low levels of EMG activity (Fig. 4). We repeated these experiments in many studies and noted the following: progesterone (SC) significantly reduces the EMG burst frequency (bursts/30 mins ± SEM:1 day treatment = 11.1 ± 1.7 *vs* controls 23.2 ± 3.5, $p < 0.01$; 2 days treatment = 10.5 ± 1.3 *vs* controls 25.4 ± 4.4, $p < 0.01$). The EMG burst amplitudes are also significantly lower in the progesterone-treated animals (μV ± SEM:1 day treatment = 74 ± 8 *vs* controls 280 ± 58, $p < 0.008$; 2 days treatment = 127 ± 31 *vs* controls 230 ± 14, $p < 0.02$). Thus, the mean burst integrals (V2) are suppressed at 1 ($p < 0.001$) and 2 ($p < 0.002$) days after P4 treatment *vs* controls, but not the burst duration ($p > 0.05$, cca. 30 seconds). This indicates that progesterone treatment suppresses uterine EMG activity and thereby inhibits birth.[76] These studies demonstrate the crucial role of myometrial inhibition in prevention of preterm delivery. The initiation of human studies on effects of various progestins on uterine EMG activity, will further address the question of myometrial inhibition by progestin treatment.

In the last 40 years, progesterone and its analogs have been administered to pregnant women in attempts prevent preterm birth and miscarriage, but with variable success. Comparing these studies is extremely difficult because they differ in terms of formulation and dose of progestin used, route of administration and timing of progestin administration.

Formulations of Progestins

Progestins are available as natural (bioidentical, micronized) progesterone and its synthetic analogs. Of the various formulations, only two have generally been considered sufficiently safe and effective to be used for prevention of preterm birth: progesterone and 17P.

Natural (Bioidentical, Micronized) Progesterone

Micronized progesterone is manufactured in a laboratory from chemicals derived from plants (Mexican wild yams and soy). It has a molecular structure identical to that of the progesterone produced in humans by the corpus luteum in the luteal phase of the menstrual cycle and during the first

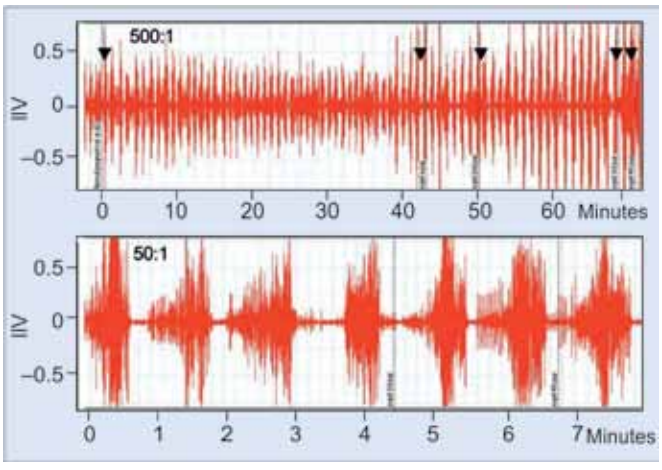


Fig. 3: Electromyography recordings from a normal pregnant rat on day 22 of gestation during delivery (arrows indicate delivery of a fetus). Recordings showing >60 minutes (top record) and for >7 minutes (bottom). Note delivery of fetuses as marked with arrows. This indicates that the animals deliver their fetuses while under anesthesia and the uterine contractions which accompany birth consists of large bursts of EMG activity. This is an example from studies using more than 20 rats

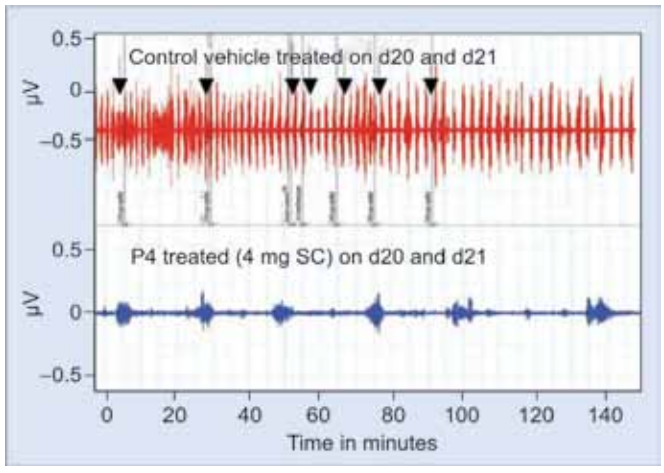


Fig. 4: Examples of EMG recordings from pregnant rats on day 22 of gestation from control rat (vehicle treated during delivery, top tracing, arrows indicate delivery of a fetus) and rat after two days treatment with P4 (4 mg micronized progesterone, P4, given subcutaneously for 2 days on day 20 and day 21 of gestation (animals not delivering). Note the difference in the bursts (amplitude and frequency) of EMG activity in control rat recording compared to the rat treated with P4. Similar differences were observed following topical treatment of P4 but not after vaginal treatment

trimester of pregnancy and (following the luteal-placental shift) in the largest quantity by the placenta. Micronized progesterone can consequently be referred to as the natural or the bioidentical, progesterone.

Safety of Progesterone in Pregnancy

In the randomized clinical trials comparing progesterone to placebo for the prevention of preterm birth, long-term infant outcomes were not evaluated.[77-79] However, natural progesterone is FDA-approved to support embryo implantation and early pregnancy, and there have been no significant adverse effects from its use in pregnancy reported to date. It should be noted that progesterone production by the placenta during pregnancy can reach levels of about 500 mg/day at term.[80]

17 Alpha-hydroxyprogesterone Caproate (17P)

This chemical is an artificially made caproate ester of 17 hydroxyprogesterone, a natural progestin produced during pregnancy in much lower quantities than progesterone. It has been developed to produce longer-lasting effects than would be available from progesterone itself.[80] The half-life of 17P is approximately 7.8 days, as compared to approximately 35 to 55 hours for progesterone.[80,81]

Physiologically, 17P is thought to have similar effects to that of progesterone. To a certain degree, this assumption is probably correct. For instance, both agents cause a secretory transformation of the endometrium.[82,83] However, there are also important physiologic differences that should be considered when deciding which agent to use in the prevention of preterm birth. Our group and others have demonstrated that 17P does not suppress myometrial contractility, whereas progesterone does.[84,85]

Safety of 17P in Pregnancy

There is evidence suggesting that the use of 17P in pregnancy is safe. In the follow-up of a single randomized trial comparing 17P to placebo for preventing preterm birth, there were no statistically significant differences in the health and development of children at 2 years of age.[86] However, there is also some data from animal and human studies suggesting that 17P may cause fetal harm by fetal toxicity (not teratogenicity). In mice, there was an increased fetal loss with 17P compared to placebo.[87] In rhesus monkeys, total embryolethality resulted following the administration of 17P at both 1X and 10X the human equivalent dose.[88] Moreover, although not statistically significant, there was an increase in intrauterine fetal death among women receiving 17P compared to placebo in the clinical trial, whose follow-up has been mentioned above.[89] An earlier meta-analysis of 17P also showed a possible, again non-statistically significant, increase in miscarriage with an odds ratio of 1.3 (0.61-2.74).[90] Further studies are needed in order to evaluate the potentially increased risk of miscarriage and stillbirth associated with the use of 17P. There are also some concerns regarding the vehicle used for 17P injections, namely castor oil. Castor oil was reported to induce labor through release of prostaglandins.[91] 17P is currently FDA pregnancy category D progestin, meaning that the FDA believes there is evidence of fetal harm.

Route of Administration

While 17P is given exclusively IM, progesterone has been administered by several routes in different studies: orally, IM and vaginally. Transdermal supplementation of progesterone to prevent preterm birth has not been studied in humans yet but it is common to use this route for application of steroids in humans.

The main advantage of oral administration of progesterone is its noninvasiveness and consequent acceptability. However, absorption of oral progesterone is quite variable, and it is rapidly metabolized by first-pass effect in the liver, which makes the oral administration essentially ineffective.[80] Moreover, side effects, such as sleepiness, fatigue and headaches, are more common when progesterone is given orally.[79] Three randomized trials to date compared oral progesterone to placebo for prevention of preterm birth. In the studies published in 1986 and 1991, oral progesterone did not prolong gestation in patients treated for preterm labor.[92,93] In 2009, in contrast, Rai et al reported a reduction in preterm delivery in women with a history of preterm birth who received oral progesterone throughout pregnancy compared to placebo.[94]

Effectiveness of IM injections of progesterone to prevent preterm birth has not been evaluated in clinical trials. The reason for this is that daily IM injections would be required to maintain therapeutic serum levels due to the relatively short half-life of progesterone. This would make this intervention very invasive, especially if progesterone was to be given by prolonged prophylactic administration to women at increased risk for preterm birth. 17P is a long-acting progestin, and can be administered once per week.[81] Even with weekly IM injections, however, side effects, such as injection side pain, swelling, itching and bruising, have been reported in up to one-third of treated women, and were more common in the progestin group as compared to placebo.[89]

The vaginal route of progesterone administration has been thought to be the preferred route when focused effects on the uterus are desired. It is noninvasive and the only side effect associated with vaginal progesterone reported in clinical studies was an increased vaginal discharge.[79] Following the concept of the liver first-pass effect after administration of oral drugs, the term 'uterine first-pass effect' was established in order to point out the minimized systemic, but optimized uterine exposure after vaginal treatment with sex steroids.[95,96] De Ziegler et al observed a 14-fold increase in the ratio of the endometrial-to-serum concentrations of progesterone after vaginal (compared to IM) administration.[95,96] However, these studies were mostly done in postmenopausal women and not during pregnancy. Volume, viscosity and pH of vaginal fluid and physical properties of vaginal epithelium largely affect the absorption of vaginally administered drugs.[97] All of these factors are significantly different in pregnant women as compared to those after menopause.

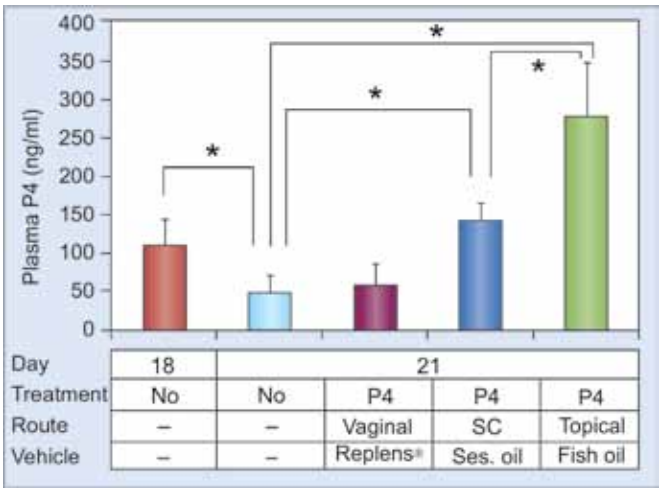
In addition, the effectiveness of vaginal progesterone seems to depend significantly on the vehicle utilized. Three randomized clinical trials in which progesterone was administered as vaginal suppositories or capsules showed a reduction in preterm delivery.[77,78,98] The exact source of progesterone was specified only in one of these three publications. Fonseca et al used of 200 mg capsules of Utrogestan®, i.e. progesterone in arachis (peanut) oil and soy lecithin.[78] On the other hand, vaginal gel (Crinone®) containing 90 mg of progesterone in a bioadhesive gel Replens®, was utilized in the two large studies which reported no benefit from vaginal progesterone.[79,99] Vaginal gel is claimed by some to have practical advantages over the capsules or suppositories. It is thought to be easier to apply and it does not liquefy. It is suggested, therefore, that it could cause less vaginal discharge, irritation and infection. Replens®, in particular, is thought to release progesterone slowly, which potentially results in sustained levels of the hormone in the uterus. However, in addition to evidence from clinical studies, our results indicate that progesterone in Replens® may not be as effective as in other vehicles. For example, transdermaly administered progesterone in fish oil delayed delivery in rats, while topical application of progesterone in Replens® did not.[73] This indicates that Replens® does not efficiently release progesterone. Furthermore, measurement of serum progesterone levels supports these conclusions (Graph 8).

More data is needed before any formulation and route of progesterone administration for prevention of preterm birth can be recommended over the others. Our study of various progestin treatments emphasizes this. Only subcutaneous injections of progesterone and transdermal administration of progesterone in fish oil (not in Replens®) delayed delivery (Graph 9).[73] Notably, none of these routes has been used in clinical trials to date. On the other hand, oral progesterone and vaginal progesterone administration, studied in humans so far, did not have any effect on time of delivery.[73]

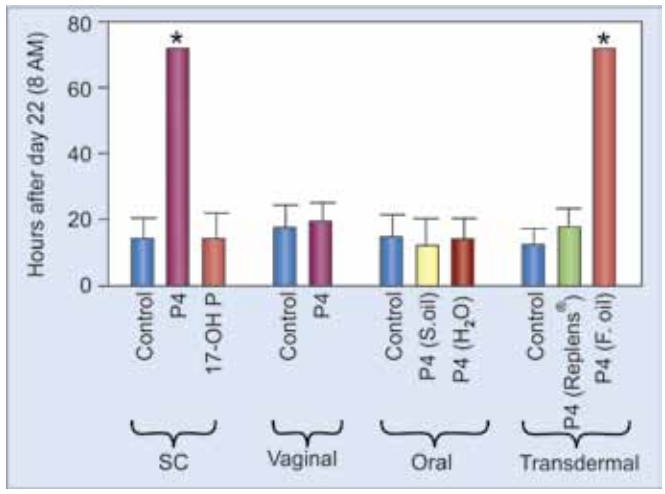
These studies clearly show that in animal models progestins with different properties have varied effects and depend upon the route of administration and vehicle.

Timing of Administration

Another reason why clinical studies of efficacy of progestins in preventing preterm birth are difficult to compare is that participants included were significantly different. The majority of randomized trials evaluated the prophylactic



Graph 8: Plasma progesterone (P4) levels in pregnant rats on day 18 and 21 without any treatment (controls) and on day 21 after treatment from day 18 until delivery with (a) vaginal P4 (15 mg, bid), (b) SC injections of P4 (4 mg), (c) topical P4 in fish oil (15 mg, bid). Asterisks indicate $p < 0.05$ compared with controls. Note the physiological P4 withdrawal from day 18 to day 21 in nontreated rats, that is prevented by SC and topical P4, but not by vaginal P4



Graph 9: The time of delivery (hours after 8 AM of day 22 of gestation) of pregnant rats treated with vehicles (controls) and progestins by different routes of administration – injections (SC; daily): vehicle: sesame oil; progesterone (P4) (4 mg); 17P (10 mg); vaginal (bid): vehicle: Replens®; P4 (15 mg, Crinone®); oral (bid): vehicle: sesame oil or H_2O; P4 (15 mg); transdermal (bid): vehicle: Replens® or fish oil; P4 (15 mg). Rats with delayed parturition were sacrificed on day 25. Asterisks indicate $p < 0.05$ compared with controls

supplementation of progestins in asymptomatic pregnant women at high risk for preterm birth. Women were considered to be at high risk for several reasons, including past history of spontaneous preterm birth or miscarriages, multiple gestation, short cervical length, cerclage in place and uterine anomalies. Earlier small trials using 17P showed mixed results. Some reported benefit from prophylactic treatment in high-risk singleton pregnancies, whereas 17P injections did not improve outcome in multiple gestations and lower-risk patients.[100-104] In 2003, two studies reinvigorated the interest in progestin treatment for prevention of preterm birth. Meis et al reported results of a large multicenter trial of 17P involving 463 women with a history of spontaneous preterm delivery.[89] Delivery at <37 weeks was reduced from 55% in the placebo group to 36% in the 17P group. Similar reduction was seen in delivery at <32 weeks, from 20 to 11%. Also in 2003, da Fonseca et al published a trial of vaginal progesterone *vs* placebo suppositories administered to 142 women found to be at high risk due to a history of previous preterm delivery, prophylactic cerclage placed or having a uterine anomaly.[77] In >90% of participants, the risk factor was previous preterm delivery. The rate of delivery at <37 weeks in treated patients was 14%, significantly less that the 29% observed in the placebo group. Delivery at <34 weeks was also lower in the progesterone group (3%) than in the placebo group (19%). However, an intervention limited almost exclusively to women who already delivered preterm is unlikely to have a substantial impact on the problem of preterm birth, since only about 10% of spontaneous preterm births occur in women with such history.[105] As a result, further studies were performed to evaluate the use of progestins in pregnant women with other risk factors. Twin pregnancies were the obvious next subject of randomized trials, given the current epidemic of multiple gestations.[106] Unfortunately, in 2007 Rouse et al showed that treatment with 17P did not reduce the rate of preterm births among women with twins, which is in agreement with the results of an earlier study published in 1980.[103,107] Recently, a large study of 500 women with twin gestation, randomized to receive either vaginal progesterone or placebo, also showed no benefit of vaginal progesterone treatment in twin pregnancies.[99] Patients who were found to have a short cervix (<15 mm) measured by transvaginal ultrasound, however, benefited from vaginal progesterone administration.[78] Da Fonseca et al reported a reduction of delivery at <34 weeks, from 34% in the placebo group, to 19% in the progesterone group. In 2009, the largest randomized clinical trial of progesterone in prevention of preterm birth to date was published. O'Brien et al evaluated the effect of vaginal progesterone in women with previous spontaneous preterm birth, and did not find any difference between vaginal progesterone and placebo groups.[79] However, further subanalysis of the data from that same trial was performed by DeFranco et al, which demonstrated that there may be a subset of women with shortened cervical length (<28 mm) for whom progesterone may have a beneficial effect in prolonging pregnancy.[108]

While many studies examined the effects of progestin prophylaxis in pregnancies considered at high risk, there is substantially less data on progestin treatment of patients following acute presentation with signs and symptoms of

preterm labor. This is unfortunate, because the majority of patients who deliver preterm do not have any risk factors.[60] There are, therefore, many apparently low-risk pregnant women who present acutely in preterm labor, and the use of progestins to prolong pregnancy and improve outcome in these patients has not been studied sufficiently. Between 1960 and 1991, four trials used progestins to stop preterm labor.[92,93,109,110] None of these showed any benefit in prolonging pregnancy. Two studies used oral progesterone, one IM 17P, and one IM MPA. The only other reported use of MPA in humans for prevention of preterm birth was in the study from Hobel et al in which MPA was also ineffective as prophylactic oral supplementation.[111] Thus, it is impossible to generalize the results of the early trials of progestin treatment for preterm labor because of their different designs. In 2007, Fachinetti et al reported a reduction of risk of preterm birth with the use of 17P as twice weekly IM injections in patients treated for preterm labor in which tocolysis was obtained with atosiban.[74] Borna et al used large doses of vaginal progesterone and showed that progesterone may be beneficial as a maintenance tocolytic, since it prolonged the latency to delivery in the treatment group as compared to patients who received no treatment.[98]

To summarize, most clinical studies on progestin treatment for prevention of preterm birth have been accomplished in patients with various risk factors who received prophylactic 17P IM or progesterone vaginally for several weeks. Since myometrial activation is an acute event, this chronic supplementation of progestins is most likely to affect cervical ripening alone. However, animal studies showed that only minor delay in the cervical changes were observed following 17P or progesterone application.[73] However, progesterone (not 17P) injections completely blocked delivery even after the process of cervical ripening was already completed. The main action must, therefore, be on the myometrium to inhibit labor. The possible benefit of progesterone inhibition of myometrial activity has, however, not been studied sufficiently in humans yet.

CONCLUSION

Despite extensive research, we are still unable to accurately predict or effectively prevent preterm delivery. In fact, current approaches to prevention and treatment of preterm labor have been shown to be disappointingly unsuccessful.[12] There is evidence from animal studies and *in vitro* studies on human tissues that supports the use of progestins for reducing the risk of preterm birth. Most of the randomized clinical trials conducted so far have evaluated prophylactic progestin supplementation in asymptomatic women throughout pregnancy. The rationale for this prolonged use is the inhibition of cervical ripening. In fact, the process of gradual remodeling of connective tissue in the cervix begins already in mid-pregnancy and is suppressed at least in part by progesterone and 17P. The use of these two compounds has been shown in some trials to be beneficial for preventing preterm birth in patients with certain risk factors, such as previous preterm birth and short cervix. Myometrial contractility is also suppressed by progesterone (but not 17P). The use of progesterone in patients presenting with signs and symptoms of preterm labor therefore seems promising due to its ability to control both cervical ripening and myometrial activity. On the other hand, the role of progesterone and other progestins in treatment of these patients has not been sufficiently studied in randomized trials yet. Additionally, effects of progestin treatments have been shown to vary extremely between different progestin formulations and different routes of administration. The optimal formulation of progestin and its vehicle, dose, and route of administration for prevention of preterm delivery remain to be determined. In our previous studies, we documented evidence that myometrial preparedness to labor and changes in cervical structure can be monitored non-invasively by measuring uterine EMG and cervical LIF.[39] These two methods objectively assess the two components of parturition: myometrial contractility and cervical ripening. They provide a methodology to evaluate various therapeutic interventions for preterm labor. In the case of progestin treatment for prevention of preterm birth, uterine EMG and cervical LIF are essential tools to obtain the critically needed comparative data on effectiveness of various progestin formulations and their routes of administration in different patients at high risk for preterm delivery. Future studies on efficacy of these treatments should, therefore, utilize the uterine EMG and cervical LIF systems.

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REVIEW ARTICLE

Antithrombotic Therapy in Patients Postcardiac Interventions

[1]Kamales Kumar Saha, [2]SK Kaul

ABSTRACT

Many primary care doctors need to know exactly how to manage antithrombotic medication of patients who have earlier undergone a cardiac intervention or surgery, if they come for a noncardiac problem. This article addresses this important issue.

Keywords: Antithrombotic management, Cardiac interventions, Cardiac surgery.

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INTRODUCTION

Cardiovascular interventions have become common in contemporary medical practice. Patients with various cardiac diseases undergo surgical or percutaneous procedures in tertiary medical care centers. Sometimes these patients may develop common, noncardiac medical or surgical problems which need to be treated by the primary care physicians or surgeons depending on the condition. So, it is important that every physician/surgeon should be familiar with common cardiac conditions and various regimens of antithrombotic therapy used in such situations.

Some common categories of such patients are: antithrombotic therapy in patients with prosthetic heart valves, antithrombotic therapy in patients of CABG, antithrombotic therapy in patients with coronary stents.

Antithrombotic Therapy in Patients with Prosthetic Heart Valves

Prosthetic heart valves are of two types—mechanical and tissue valves. Tissue valves are usually used in elderly patients as most tissue valves have limited life span. Mechanical heart valves are used in younger patients but require lifelong anticoagulation therapy.

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Tissue Valves

These valves are made from biological materials usually bovine or porcine. Mitral biological valves have stents. Aortic tissue valves may be stented or stentless. Valves made from human cadavers are called homografts and are mostly used in aortic position. Tissue valves have limited life span and are used in patients more than 65 years of age. Degeneration of tissue valves is slower in older patients. It should not be used in young or adolescent patients as there may be accelerated degeneration.

Advantage of Tissue Valves

These valves do not require long term anticoagulation. Some authorities advocate 3 months of anticoagulation in patients of mitral bioprosthesis. Anticoagulation is not required in patients with stentless aortic valves or aortic homografts. A few surgeons advocate 3 months of anticoagulation in patients with stented aortic vlave while others think only aspirin is enough for patients with aortic bioprosthesis.

Disadvantages of Tissue Valves

All tissue valves have finite life. In elderly patients, rate of degeneration is slower. Many patients with tissue valve may require reoperation after 5 to 15 years. Moreover, tissue valves are more expensive than mechanical valves. Reoperation risk has to be explained to patients before implanting tissue valve as many patients may not want second surgery due to fear and/or economic reasons.

Mechanical Valves

These valves are made up metal or synthetic materials. These valves last throughout a patient's lifetime. Patient has to be on anticoagulant for the rest of his/her life. Oral anticoagulation is preferred. Level of anticoagulation is monitored by prothrombin time (PT) and international normalized ratio (INR) estimation. Target level of therapeutic INR in patients of prosthetic heart valves is between 2.0 and 3.0.

Oral anticoagulant agents are vitamin K antagonists (like warfarin and acecoumarin). So, any food which contains high amount of vitamin K will decrease their effectivity. Vitamin supplements containing vitamin K will have similar effect. Large amount of vitamin K rich food can lead to

thrombotic complication. Foods rich in vitamin K include the following: green leafy vegetables, kiwifruit, grapes, avocado, cheese, soybean oil, egg yolk. These foods are to be avoided by patients taking oral anticoagulants.

Surgical Procedure in Patients on Anticoagulants

Elective Surgery

Patient should be admitted 3 days prior to elective procedure. Oral anticoagulant should be stopped. Intravenous heparin is to be started with estimation of clotting time (CT) before each dose. Usually to start off, injection heparin 5000 units is given every 6 hourly.[1] If CT is more than 10 minutes, interval of dosing is to be increased. Once INR is less than 1.5, elective surgery can be performed. Heparin is stopped 4 to 6 hours prior surgery. Postoperatively, heparin can be restarted depending on the comfort level of the operating surgeon.[2] Patient has to be monitored in intensive care unit for prosthetic valve function till therapeutic INR is achieved.

Emergency Surgery

Patient should be transfused fresh frozen plasma (FFP) to bring the INR to less than 1.5 and once this is achieved surgery can be performed safely. Postoperative management remains similar to elective surgery. Injection vitamin K should be avoided and FFP should be used liberally. In life-threatening bleeding injection, vitamin K may be used in reduced doses. Once vitamin K is used, patient may become refractory to oral anticoagulant for an unpredictable period of time.

Pregnancy in Patients with Heart Valve

In first and third trimester, patients may be switched to heparin,[3] unfractionated heparin (UFH) or low molecular weight heparin (LMWH). There is added risk to fetus if oral anticoagulation is continued in first trimester. Some clinicians advocate continuation of oral anticoagulation till the time of delivery when patient is switched to heparin.[3] Child birth can be managed in a fashion similar to the protocol for elective surgery noted above.

Antithrombotic Therapy in Patients having undergone CABG

Conventionally, patients are put on aspirin after CABG. Recent meta-analysis suggests dual antiplatelet therapy improves graft patency[4] after CABG. This meta-analysis analyzed five randomized controlled trials and six observational studies involving 25,728 patients. The conclusions of the meta-analysis as follows:

A. Early SVG occlusion rate was reduced with dual antiplatelet therapy ($p = 0.02$).
B. In hospital, 30-day mortality was lower with aspirin and clopidogrel ($p < 0.0001$).
C. In a pooled analysis of studies involving off-pump CABG compared to aspirin alone, dual antiplatelet therapy reduced the risk of perioperative myocardial infarction and saphenous vein graft occlusion by 68 (47 to 71%) and 55% (2 to 79%) respectively.

Moreover, patients of CABG are prone to deep venous thrombosis (DVT) and should receive DVT prophylaxis as per institutional protocol. Surgical intervention in patients with antiplatelet therapy is discussed later.

Antithrombotic Therapy in Patients with Coronary Stents

Dual antiplatelet therapy is routine after stents. Whenever a patient who has undergone a coronary angioplasty and stenting or CABG goes for a noncardiac surgery (NCS), the dilemma is whether to stop antiplatelet agents or continue them in the perioperative period. Cessation of antiplatelet therapy in the perioperative period can lead to rebound thrombotic phenomenon which can result in stent or graft thrombosis that may be fatal.[5] At the same time, continuing antiplatelet therapy in the perioperative period may pose a threat of increased risk of bleeding. American College of Cardiology recommends that elective noncardiac surgery should be ideally delayed for at least 12 months in case of DES and 1 month in case of a bare metal stent.[6] There are no available recommendations for patients who have undergone CABG. However, if an emergency surgery has to be performed, there are basically three possible scenarios of temporary withdrawal of oral antiplatelet drugs for planned NCS according to bleeding risk of surgery.[7]

1. Surgery with low bleeding risk, e.g. cataract surgery, oral dental surgery, etc. Interruption of oral antiplatelet therapy is not necessary, irrespective of the ischemic risk profile. Continue both aspirin and clopidogrel.
2. Surgery with intermediate bleeding risk, e.g. GI surgeries, cholecystectomy, appendicectomy, etc. Continue aspirin during perioperative period. Stop clopidogrel 5 days prior to surgery with reintroduction as soon as possible.
3. Surgeries with high bleeding risk, e.g. intracranial surgeries, prostate surgery, aortic surgery, ENT surgeries and surgery in posterior segment of eye. Stop aspirin and clopidogrel 7 days before planned surgery and substitute with alternative antithrombotic therapies. Regular treatment should be resumed as soon as possible after surgery.

Alternative antithrombotic therapy has revolutionized in recent times with introduction of short acting potent anti-

platelet agent tricagrelor. Clopidogrel and aspirin can be stopped and patient can be switched to tricagrelor 90 mg twice daily. This can be stopped two days before planned surgery and patient can be put on LMWH in a dose of 85 to 100 iu subcutaneously 12 hourly. After the surgery is performed LMWH can be restarted as early as 6 hours (range 6-24 hours depending on the type of surgery, risk of bleeding and risk of thrombotic complications and the comfort level of surgeon). Preoperative antiplatelet therapy may be resumed after 48 hours or more when there is no risk of bleeding.

CONCLUSION

Antithrombotic therapy is lifesaving in patients with cardiac interventions. However, in certain clinical conditions, this can be double-edged sword when there is a high risk of bleeding and thrombosis. Attempts to reduce bleeding are associated with increased risk of thrombotic complications. Balancing both ends of spectrum with proper antithrombotic strategy and individualized approach is essential in such tricky situations.

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CASE REPORT

Rare Presentation of Large Cell Lymphoma

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ABSTRACT

Lymphoma is a common cancer of childhood. Its pathologic subtype of anaplastic large cell Non-Hodgkins Lymphoma (NHL) is rare and spread to the central nervous system (CNS) is even rarer. We present here such a case who presented to us with an acute history of fever and diplopia and co-existent polyarthritis since 8 years diagnosed as NHL with CNS involvement. CNS involvement is a rare presenting manifestation of NHL and is an important cause of morbidity and mortality in these patients. Chronic arthritis could be a risk factor or manifestation of immunodeficiency or immune dysregulation when associated with lymphoma. It is important to suspect NHL in children presenting with these symptoms for prompt evaluation and better clinical outcome.

Keywords: Pediatric lymphoma, Anaplastic large cell lymphoma, ALK-1.

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INTRODUCTION

Lymphoma is the third most common cancer among United States children.[6] Of its two broad categories, Hodgkins Lymphoma (HL) and Non-Hodgkins Lymphoma (NHL), NHL is 1.5 times more common and affects boys three times as frequently as girls.

The clinical presentation of NHL depends upon the site, extent and pathologic subtype of the tumor. In the head and neck region it manifests as painless lymph gland enlargement; in the abdomen as mass or intestinal obstruction; in the Central Nervous System (CNS) as increased intracranial pressure. In the chest it usually arises in the anterior mediastinum leading to compression of the airways and superior vena cava.

The four major pathologic subtypes are Burkitt lymphoma (BL), lymphoblastic lymphoma (LL), diffuse large B cell lymphoma (DLBCL) and anaplastic large cell lymphoma (ALCL). About 70% of cases of ALCL are of T cell origin, 20% are of null cell origin and 10% of B cell origin. Patients with ALCL commonly have a t (2; 5) translocation (90%), which results in the formation of a fusion gene encoding the constitutively active NPM-Activin receptor like kinase 1 (ALK 1) tyrosine kinase.

ALCL manifests either as a primary cutaneous manifestation (10%) or as systemic disease (fever, weight loss) with dissemination to liver, spleen, lung, mediastinum or skin; spread to the bone marrow or CNS is rare.[1,2] Secondary spread of primary nodal or systemic ALCL to the CNS is also unusual. Till date in literature only 15 cases of primary ALCL of CNS have been reported.[1,2,4,5]

CASE REPORT

A 16-year-old boy presented to us with intermittent low grade fever from 2 weeks, headache and double vision in left eye since 2 days and pain in the back and right leg since 1 day. Possible infective or inflammatory etiology with raised intracranial tension, e.g. viral encephalitis, vasculitis, DVT with embolization, trauma with fat embolism, thromboembolism was suspected. Our patient did not have symptoms of dyspnea, rashes, convulsions, vomiting and trauma. The child also had complaints of swelling, pain and restriction of movement in knees, ankles and multiple small joints of both lower limbs since the age of 6 years for which he had multiple orthopedic consultations and had received multiple medications but was never relieved. At the onset of the joint complaints child was diagnosed as a case of tuberculosis (TB) of left knee joint based on synovial fluid TB Polymerase Chain Reaction and had received anti-tuberculosis treatment for 6 months as per medical records. Physical examination at presentation to us showed a well-grown child with a pulse rate of 96 per minute with normal rhythm, volume and character; respiratory rate of 22/min and BP of 120/72 mm Hg. He had no pallor, cyanosis, edema or significant lymphadenopathy at any of the lymph node areas and no calf tenderness. He had inflamed small joints of all 4 limbs as well as both ankles.

He was conscious, well behaved child, with intact higher functions. There was evidence of left lateral rectus palsy (6th cranial nerve); other cranial nerves were normal and no evidence of papilledema. Nutrition was normal. Power and tone were normal in all the joints and groups of

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muscles. Deep tendon reflexes were brisk. Babinski's sign was positive on both the sides. Cerebellar functions were normal and there were no signs of meningeal irritation. There were decreased breath sounds on right inframammary area without any adventitious sounds. There was no evidence of any organomegaly. With these findings, the initial provisional diagnostic possibilities considered were systemic onset juvenile rheumatoid arthritis, reactivation of tuberculosis with multi system involvement, histiocytosis and macrophage activating syndrome. The child had a normal hemoglobin, raised total leukocyte counts (21,830 per cu mm) with neutrophilic predominance (83%) and raised platelets (6,93,000 per cu mm), ESR of 28 mm at the end of 1 hour. Liver and kidney function tests were normal. There were nodular lesions in the right para hilar regions on chest X-ray. Contrast enhanced computed tomography (CECT) of chest showed mildly enhancing anterior mediastinal mass of 8.4 × 5.6 cm with a radiological differential diagnosis of thymic lesion or nodal mass, with sub centric nodes in pre and paratracheal region, subcarinal region with few subpleural subcentimeric opacities in bilateral lung fields (Fig. 1). On contrast enhanced scan of brain there were symmetrical areas of hyperintensity on T2 weighted imaging and FLAIR involving bilateral paramedian thalami with restricted diffusion on Diffusion weighted Imaging suggestive of encephalitis or infarcts (Fig. 2). Bone marrow aspirate was normocellular with increase in myeloblastic, megaloblastic, plasma cell numbers. Cerebrospinal fluid analysis was normal, lupus anticoagulant was absent, direct coombs test was negative, serum ferritin, plasma fibrinogen were in normal limits. The child was referred to pediatric oncology center for further management. CT guided biopsy of anterior mediastinal mass was carried out. Histopathology confirmed the diagnosis of ALCL. Immunohistochemistry showed T-cell immunophenotype with tumor cells immunopositive for CD 30, CD 3, ALK-1, Mic-2 and LCA and immunonegative to CD-34, CD-20, Pax-5 and CD-15. Positron Emission Tomography - CT whole body showed avid FDG uptake in the bilateral thalamic regions, pituitary fossa, in the spinal cord at the level of C1, in the prevascular, anterior mediastinal, right internal mammary, bilateral hilar and subcarinal nodes, right deep pectoral and left axillary adenopathy, nodular infiltrate in both lungs predominantly the lower lobes, perigastric, peripancreatic, periportal adenopathy, diffuse gastric wall thickening, pulmonary nodules suggestive of high grade lymphomatous involvement along with soft tissue deposits along the nerve roots exiting from the sacral foramina secondary to lymphomatous involvement. With the presence of generalized lymphadenopathy, a final diagnosis of secondary ALK-positive ALCL with CNS involvement was made. Patient was to be started on chemotherapy but before that child landed up in hypovolemic shock due to severe upper gastrointestinal bleeding and succumbed to the same. No postmortem was carried out and cause of death was given as hypovolemic shock due to upper gastrointestinal bleeding in a child with stage 4 non-Hodgkin's lymphoma.

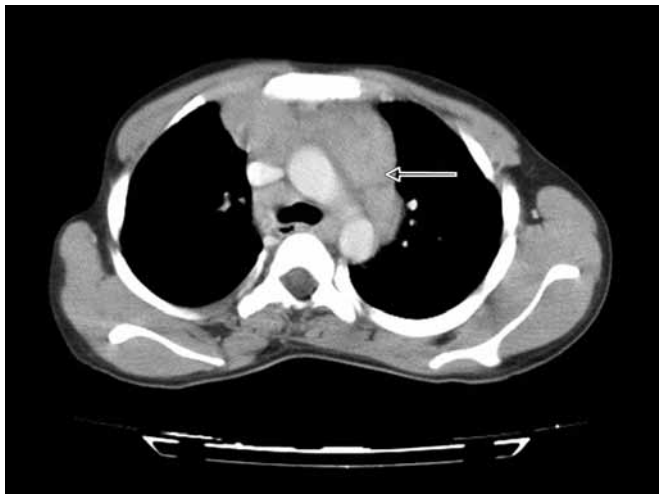

Fig. 1: CECT chest showing mildly enhancing mass lesion in anterior mediastinum extending to the prevascular region

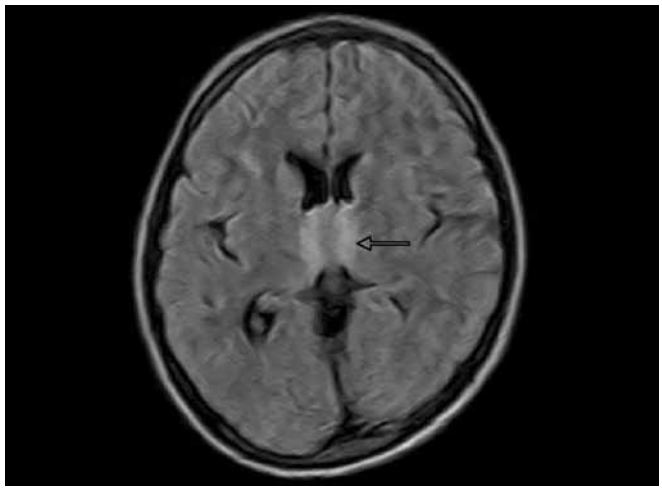

Fig. 2: MRI brain: Fluid attenuated inversion recovery sequence (FLAIR) image showing bilateral paramedial thalamic hyperintensity

DISCUSSION

ALCL accounts for approximately 3% of adult and 10 to 15% of childhood non-Hodgkins lymphoma.[3] It frequently involves both nodal and extranodal sites. Extranodal sites which are most commonly involved are skin, bone, soft tissue and liver.[3] Primary CNS lymphomas are rare, mostly represented by diffuse large B cell lymphoma. Till date in literature only 15 cases of primary ALCL of CNS have been reported.[1,2,4,5] CNS is rare site to be involved by systemic ALCL. Kaplinsky et al[4] reported a 2-year-old boy with generalized lymphadenopathy, skin involvement, hepatosplenomegaly and pulmonary infiltrates along

with CNS involvement; our case did not have cutaneous manifestations. A case reported by Karikari et al[5] was a primary ALK-1 positive ALCL who developed cervical lymphadenopathy 3 weeks after the diagnosis of primary CNS lymphoma. In their report, they have discussed the possibility of a primary nodal lymphoma with metastasis to the brain.

ALK-1 positive ALCL is a clinically aggressive lymphoma. However, overall survival and longer disease free survival is observed after treatment with aggressive chemotherapy. Patients affected by ALK-1 positive ALCL have a significant better overall survival than ALK-1 negative ALCL patients (5-year overall survival: 70 to 80 *vs* 33-49%).[7,8] It is still controversially discussed if this observation can be explained by the biological role of the ALK-1 fusion proteins or by the younger age of ALK-1 positive ALCL patients. Another unexplained manifestation in our case was the long standing history of polyarthritis which had been unresponsive to the antituberculous treatment that the child was given at the outset and the presence of arthritis at presentation to us. The PET CT did not show any joint or bone uptake. So it is unlikely that the arthritis was because of lymphomatous etiology. Systemic onset juvenile idiopathic arthritis is known to get complicated with macrophage activating syndrome and hemophagocytic lymphohistiocytosis. Autoimmune conditions like arthritis and lymphomas are found in primary immunodeficiency disorders like hyper IgM syndromes, autoimmune lymhoproliferative syndrome. But there were no other autoimmune manifestations other than arthritis in favor in our patient. Immunological workup for immunodeficiency was not done for this child.

Gastrointestinal bleeding is a known complication of non-Hodgkin's lymphoma. The PET scan in our patient showed diffuse gastric wall thickening. The platelet count at this stage was normal. Local pathology bleeding profusely into the GI tract with resultant hypovolemia caused death of the child. This exceptionally unusual case widens the differential diagnosis of mediastinal mass in childhood and emphasizes that thorough workup and clinical examination are the most important for early diagnosis and treatment.

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CASE REPORT

Complicated Hyperuricemia

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ABSTRACT

Gout is most common arthritis in men and its prevalence increases with increased life expectancy. Untreated hyperuricemia lead to complication like renal impairment, hypertension, diabetes, cardiovascular complication. We are reporting here such a case of untreated hyperuricemia. A 45 years old male presented with polyarthritis having since 3 years. He is also a known case of hypertension and diabetes for last 6 months. Patient is on steroid for last 1 year. Patient presently has bilateral pedal edema, swelling of both knee joints, bilateral 1st metatarsophalangeal joint and tophaceous swelling in different areas of the bodies. On blood examination-raised serum uric acid, creatinine, blood urea level. Urine shows uric acid crystal and albuminuria. Synovial fluid shows uric acid crystals. Long-term untreated hyperuricemia associated with hypertension and cardiovascular and renal diseases. Therefore raised uric acid lead to multiple system disease involvement.

Keywords: Gout, Hyperuricemia, Polyarthritis.

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INTRODUCTION

Hyperuricemia, defined as a serum urate concentration higher than the limit of urate solubility (408 μmol/l or 6.8 mg/dl at 37°C, pH 7.4)[1] and manifest clinically as gout. Gout is the most common arthritis in men and its incidence rising with age.[2] Untreated hyperuricemia lead to chronic tophaceous gout associated with renal impairment diabetes mellitus, hypertension, cardiovascular complication.[3] We are reporting here such a case of untreated hyperuricemia.

CASE REPORT

A total of 45 years old male presented with polyarthritis, since 3 years. He is also a known case of hypertension and diabetes mellitus for last 6 months. Patient is on steroid for last 1 year. Patient presently has bilateral pedal edema (Fig. 1), swelling of both knee joints (Fig. 2), and tophaceous swelling on right elbow joint (Fig. 3), bilateral 1st metatarsophalangeal joint and dorsum of left foot (tarsal joint) (Fig. 4).

On blood examination—serum uric acid is 12.2 mg/dl, creatinine 6.2 mg/dl, blood urea 221 mg/dl, urine show uric acid crystals (Fig. 5), albumin^{++}, pus cells 45-50/hpf.

Synovial fluid shows uric acid crystals suggestive of gouty fluid.

Ultrasonography shows bilateral nephrocalcinosis.

DISCUSSION

Uric acid levels also vary significantly within humans as the result of factors that increase generation (such as high purine or protein diets, alcohol consumption, conditions with high cell turnover, or enzymatic defects in purine metabolism) or decrease excretion. A reduction in glomerular filtration rate (GFR) increases serum uric acid, although a significant compensatory increase in gastrointestinal excretion occurs.[9] Hyperuricemia also may result from increased net tubular absorption. After filtration, uric acid undergoes both reabsorption and secretion in the proximal tubule, and this process is mediated by a urate/anion exchanger and a voltage sensitive urate channel.[10,11] Organic anions, such as lactate decrease urate secretion by competing for urate through the organic anion transporter, whereas several substances, including probenecid and benziodarone, have opposite effects.[12] Hyperuricemia is usually defined as 6.5 or 7.0 mg/dl in men and 6.0 mg/dl in women. Uric acid is higher in men and postmenopausal women because estrogen is uricosuric.[13] If the underlying hyperuricemia remains without sufficient therapy for years, chronic tophaceous gout may manifest. In addition to other comorbidities, such as hypertension, diabetes, and cardiovascular disease, approximately every second patient with gout has some degree of renal impairment, defined by an estimated glomerular filtration rate (eGFR) of less than 90 ml/min per 1.73 m^2 as per Cockroft-Gault equation corrected for ideal body weight.[3] In subjects with obesity, insulin resistance and dyslipidemia (the metabolic syndrome), hyperuricemia frequently occurs because insulin stimulates sodium and urate reabsorption in the proximal tubule.[13]

Coexistence of chronic severe tophaceous gout and renal insufficiency poses significant therapeutic challenge. Uric acid is increased in subjects with renal disease as the result

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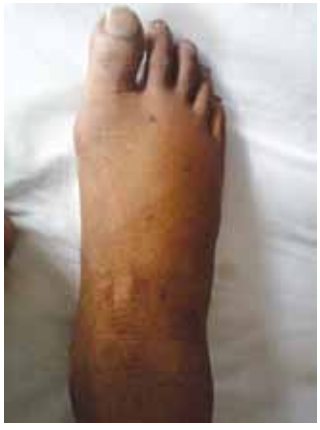

Fig. 1: Tophaceous swelling of bilateral first metatarsophalangeal joint and dorsum of left foot (tarsal joints)

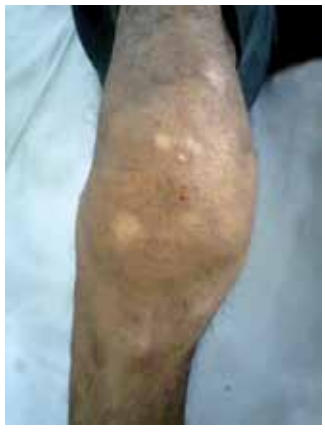

Fig. 2: Swelling of knee joints

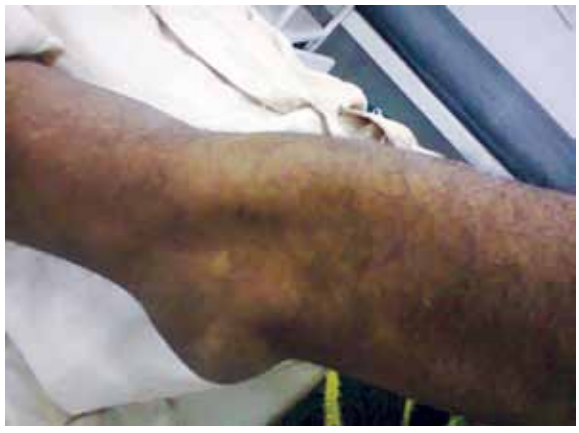

Fig. 3: Pedal edema

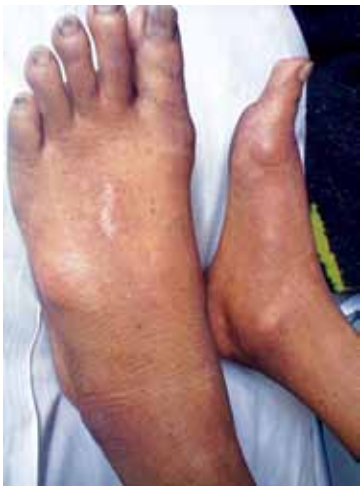

Fig. 4: Tophaceous swelling of right elbow joint

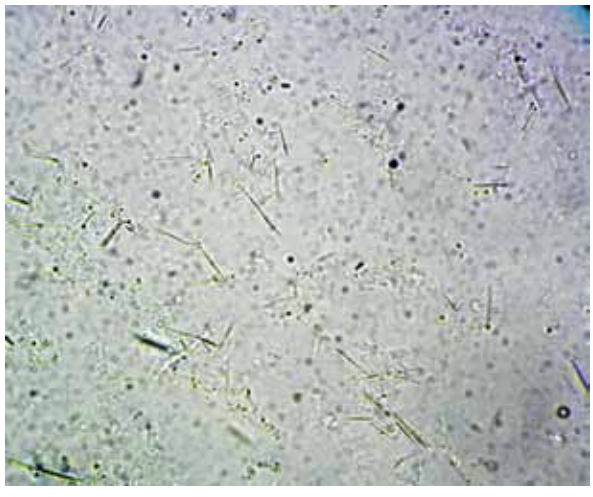

Fig. 5: Synovial fluid microscopic appearance showing needle shaped monosodium urate crystal

of reduction in GFR and renal urate excretion. Diuretics, such as thiazides, increase serum uric acid by stimulating both sodium and urate reabsorption in the proximal tubule. Alcohol intake results in elevated uric acid levels due to increased urate generation (from increased adenine nucleotide turnover) and decreased excretion (due to lactate blocking tubular transport of urate).[14,15]

Hypertension is present in 25% of untreated hypertensive subjects, in 50% of subjects taking diuretics, and in 75% of subjects with malignant hypertension.[4,5] The increase in serum uric acid in hypertension may be due to the decrease in renal blood flow that accompanies the hypertensive state, since a low renal blood flow will stimulate urate reabsorption.[6] Hypertension also results in microvascular disease, and this can lead to local tissue ischemia.[7] In addition to the release of lactate that blocks urate secretion in the proximal tubule, ischemia also results in increased uric acid synthesis.[8] With ischemia, ATP is degraded to adenine and xanthine, and there is also increased generation of xanthine oxidase. The increased availability of substrate (xanthine) and enzyme (xanthine oxidase) results in increased uric acid generation as well as oxidant (O_2^-) formation.

Tissue ischemia and activation of platelet due to hyperuricemia leads to endothelial dysfunction and oxidative stress in conditions, such as heart failure and diabetes.

CONCLUSION

As raised uric acid leads to multiple system disease involvement and hyperuricemia is easily treatable, so it must be investigated in every patient with hypertension, diabetes, renal and cardiovascular diseases.

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