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Ruhuna Journal of Medicine

The Official Journal of the Faculty of Medicine
University of Ruhuna Galle, Sri Lanka

http://www.medi.ruh.ac.lk/index.php/research/publicationshome

July 2016 *Volume 04* *Number 01*

Astercantha longifolia - "නීරමුල්ලි" ; First ever Patent to Faculty of Medicine

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Ruhuna Journal of Medicine

Proceedings of Faculty of Medicine Academic Session (FMAS) -2016, University of Ruhuna

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Cover story

The research project conducted by Dr. Ruwani Hewawasam, Senior Lecturer in Biochemistry, for her MPhil thesis secured the first ever "Patent" to the Faculty of Medicine, University of Ruhuna. The patent titled "**Aqueous plant extracts used in hepatoprotective and antioxidative activities**" awarded to Dr. Ruwani Hewawasam, Prof. Kamani Jayatilaka and Prof. Chitra Pathirana by the National Intellectual Property office of Sri Lanka in July 2013 covered five plant extracts used for the study. *Asteracantha longifolia* commonly known as "*Neeramulli*" is one of the five plants they had investigated. The team of researchers has shown that the aqueous plant extract possesses hepatoprotective and antioxidative activity against carbon tetrachloride and paracetamol induced hepatocellular damage in mice[1,2]. Further, they have confirmed that the prophylactic consumption of the plant extract has more protective effect than the therapeutic consumption. Study continues in order to elucidate the clinical effectiveness and the mechanism of action of the most effective plant extracts identified in the study. *Asteracantha longifolia* is a delicacy in villages as a curry/sambol and is commonly used in ayurvedic medicine in Sri Lanka in the treatment of liver diseases, as a diuretic and an aphrodisiac.

[1] Hewawasam RP, Jayatilaka KAPW, Pathirana C and Mudduwa, L.K.B. Protective effect of *Asteracantha longifolia* extract in mouse liver injury by carbon tetrachloride and paracetamol. *Journal of Pharmacy and Pharmacology* 2003; 55(10): 1413-8.
[2] Hewawasam, R.P., Jayatilaka, K.A.P.W. and Pathirana, C. Protective effect of Asteracantha longifolia against carbon tetrachloride and paracetamol induced oxidative stress and lipid peroxidation in mice. *Journal of Pharmacognosy and Phytotherapy* 2016; 5(5): 179-183.

Photograph & Cover page designed by Dr. Channa Yahathugoda

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Foreword

It is with great honor that I send this message to the Faculty of Medicine Academic Sessions (FMAS), University of Ruhuna. On behalf of the organizing committee it is a great pleasure to welcome you to the fourth FMAS.

FMAS was formed in year 2013 with a sense of gaining international recognition and uplift the research environment in the Faculty. In designing this year programme, the aim was broaden to integrate biomedical research with clinical care. Hence, the theme of this year is "Biomedical research and improved clinical care".

I extend my sincere appreciation to three world renowned expert panel for sharing their expertise with us in the Toxicology symposium. Finally, I thank presenters, all those who sent abstracts of their scientific work, reviewers, members of the FMAS committee and all those who contributed for the success of this event.

I wish you enjoy the scientific programme.

Dr. S.S. Jayasinghe
Chairperson

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Message from the Vice Chancellor

First of all on behalf of University of Ruhuna, I as the Vice Chancellor, extend my warmest congratulations and sincere thanks to the Dean of the Faculty of Medicine, Chairperson and the members of the organizing committee for organizing Faculty of Medicine Academic Sessions for the 4th consecutive year. Therefore I send this message with great pleasure for the proceedings of the FMAS – 2016. I firmly believe this event will be an intellectual platform for academics, scholars, researchers and practitioners from diverse domains of medicine. Further I believe Ruhuna Journal of Medicine (RJM) will be a significant source of up to date information about medical research in the country. I suppose that the proceedings of the FMAS – 2016 which will be published in RJM will reflect the breadth of the research being conducted by our academics in the medical field.

As the Vice Chancellor, I am proud that our academics have continuously worked toward raising the bar in terms of quality and depth of research done and broadening the scope of the research work to ensure maximum impact across various subject areas. We have much to be proud of a record of research in a number of areas that have impacted positively on the development of the country, our graduates have gone on to serve at the highest levels within and outside the country and University of Ruhuna has grown in terms of student enrolment, staff complement and visibility among the universities in the country as well as in Asia. There is much more to be done, but I do believe that we have now established a strong base from which we can continue to develop. As University of Ruhuna looks at its future, particularly in times of limited financing and increased competition, Faculty of Medicine Academic Sessions and RJM must serve as important platforms to cultivate even more vibrant research culture within our academics. My warmest congratulations again!

Professor Gamini Senanayake
Vice Chancellor,
University of Ruhuna

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Message from the Dean

I am most happy to send his brief message and best wishes to the 2016 Annual Academic Sessions of the Faculty of Medicine. This event started in 2013 is the major academic event organized by the Faculty.

We have seen an increase of research publications from the Faculty members during the recent past. A simple search in the PubMed using the phrase "Faculty of Medicine, Ruhuna" yielded 22 publications in the year 2015 and this contrasts with 9 publications in the year 2014. I may have missed some publications as the search was not 100% sensitive.

Faculty of Medicine, University of Ruhuna is committed to research and many academics are involved in research in selected areas. It is important for a researcher to remain focused in the selected field of research in order to make advances and also to gain recognition among academia.

This year organizers have made few changes to the programme to make it more effective. I take this opportunity to thank the Chairperson and the committee for the commitment and dedication shown to make this year academic sessions a success. Finally, I wish all the success for this year Academic sessions and hope that participants will find it academically rich and informative.

Professor Sarath Lekamwasam
Dean,
Faculty of Medicine

A randomized controlled trial of rectal analgesia with diclofenac sodium for relief of perineal pain following child birth

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ABSTRACT

To evaluate rectal diclofenac sodium in the relief of perineal pain after trauma during childbirth a randomized, double blind trial was conducted. Women with an episiotomy or lesser or equal perineal tears to second degree including vestibular tears, which required suturing of the Obstetric Department (Ward 21), Professorial Unit, Colombo South Teaching Hospital, Kalubowila were enrolled.

Women were randomly allocated to either diclofenac sodium or placebo suppositories (Anusol) , using a random – number table. Treatment packs contained two, diclofenac sodium 100 mg and diclofenac sodium 50 mg suppositories or two placebo suppositories, The first (diclofenac sodium100mg or placebo) was inserted when suturing was completed , and the second (50 mg diclofenac sodium or placebo) 12 hours after birth. Women were asked to indicate their degree of perineal pain with different activities (resting/ walking/ sitting and squatting) 24 hours after birth , using visual analogue scale Main outcome measure was overall pain score at 24 hours after birth.

A total of 169 women were recruited, with 84 randomized to diclofenac sodium suppositories and 85 to placebo. Women in the diclofenac sodium group were significantly less likely to experience pain within 24 hours of delivery (percentage of mean pain score reduction , 45% , $P < .001$) with different activities compared placebo.

The use of rectal diclofenac sodium is a simple and effective method of reducing the pain experienced by women following perineal trauma within the first 24 hours after childbirth .

This study was done in Colombo South Teaching Hospital as a partial requirement for MD in Obstetrics & Gynaecology in March 2008. Results were included in a thesis with one published paper in Sri Lanka Journal of Obstetricians and Gynaecologists. Abstract presentation was done in annual scientific session of Sri Lanka College of Obstetricians & Gynaecologists.

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Introduction

Perineal damage can cause significant maternal morbidity both immediate and long term. Morbidity associated with child birth may affect a woman's physical, psychological and social wellbeing. A vast majority of morbidity associated with perineal trauma is under reported [1]. Perineal discomfort may disrupt breast feeding, family life and sexual relationship [2]. Majority of mothers experience perineal pain and discomfort for 10 to 12 days following childbirth, which is more severe in the immediate postnatal period and some of them will continue to have long term pain for 3 to 8 months following delivery [1,3]. Other long term morbidities associated with perineal trauma is superficial dispareunia, fecal incontinence and some degree of urinary incontinence [3, 4,5]. Trauma to perineum is very painful and distressing to mothers and it has been associated with restricted mobilization, acute urinary retention, constipation, disturbance in breastfeeding etc.

Vast majority of perineal trauma is due to intentionally made episiotomy to facilitate vaginal delivery. Episiotomy rates vary considerably in various countries according to individual practices and policies of staff and institutions. Overall rates of episiotomy in different countries range from 8% to 99% [6, 7]. In Sri Lanka almost all primipara and most of the multipara experience episiotomy in hospital practice.

Postpartum pain relief is one of the ignored aspects following childbirth due to under estimation. As almost all mothers experience perineal pain in differing severity following child birth, the provision of safe and effective pain relief in modern healthcare practice is an essential component in obstetrics as

mothers do not accept perineal pain following childbirth. Currently in Sri Lanka, there is no routine practice of perineal pain relief protocol, following child birth.

The provision of pain relief for perineal trauma has several therapies in clinical practice, which include rectal analgesics, oral analgesics, local anaesthetics, parenteral analgesics, therapeutic ultrasound and non pharmacological applications such as baths and ice packs [8]. Simple oral analgesics such as paracetamol and paracetamol + Codeine can be used as pain relief for mild pain [9]. But efficacy is poor for moderate to severe pain following more extensive trauma such as 3rd and 4th degree perineal tears. Simple analgesics have a place when combined with other pharmacological agents for additive effect [10]. Non-Steroidal-Anti-Inflammatory-Drugs (NSAID's) are important analgesics for mild to moderate pain especially associated with physical trauma to tissue with acute inflammation [11]. A wide range of drugs are available with various efficacies administered in different routes; local/oral/rectal/parenteral. Local infiltration of anesthetic agents such as lignocaine has a limited place for perineal pain relief due to its short lasting activity. Parenteral agents such as morphine and pethidine are associated with unwanted side effects e.g. drowsiness, respiratory depression, difficulty in breastfeeding etc. Therefore, these agents are not used for routine perineal pain relief.

In general, medical care, the rectal route of analgesics administration has been favoured with good compliance when oral preparations cause gastric irritation, nausea and vomiting [12]. As the rectal mucosa have a rich vascular, and lymph supply, absorption of drugs is fast and analgesic effect occurs in a shorter period than oral route. Compared to oral administration of drugs, first pass metabolism is avoided in rectal administration [11].

Rectal diclofenac sodium is an effective, cheap, widely available and safe analgesic agent for pain relief. Studies assessing the efficacy of rectal analgesics in post-operative pain relief have indicated significant reduction of pain experienced [12], and reduced requirement of additional analgesia [13,14], although evidence of efficacy and safety of rectal analgesics in perineal pain relief is lacking [15]. Aim of this study was to evaluate rectal diclofenac sodium in relief of perineal pain after trauma during childbirth.

Objective

To evaluate rectal diclofenac sodium in the relief of perineal pain following trauma within first 24 hours after childbirth.

Material and Methods

A randomized, double blind controlled trial was carried out in the Obstetrics department, Professorial Unit, Colombo South Teaching Hospital (CSTH), Kalubowila, from 1st of October 2005 to 1st of February 2006. Ethical approval was obtained from Ethics and Research Committee of CSTH.

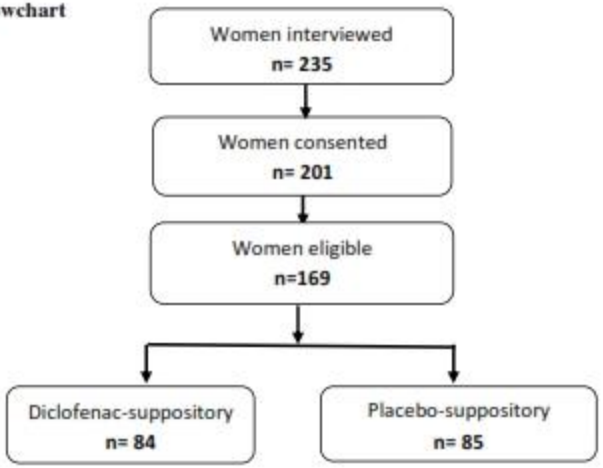
The minimum sample size calculation was based on previous research carried out by Corkhill *et al.* (2001) [16], which showed a mean pain score of 43.5 (SD 21.8) using 101-point numerical rating scale for perineal pain, with rectal analgesia, to detect a clinically significant reduction in pain score from 43.5 to 32.6 in visual analogue scale). It was necessary to recruit 168 subjects to trial (5% level of significance with 90% power).

Study information was provided to potentially eligible women with the period of gestation greater than 37 weeks admitted to the unit for vaginal delivery after obtaining their informed consent. Eligible women were randomly allocated following delivery to either diclofenac sodium group or placebo suppository group using random number table. The randomisation schedules were prepared by a researcher not involved in patient care using random number tables. Perineal repair or episiotomy suturing were done by senior registrar or registrar or house officer using polyglycolic acid (vicryl) by subcuticular suturing technique. Treatment pack contained 100mg and 50mg diclofenac sodium, and placebo (Anusol suppositories, which was having least analgesic action in the perineum), 1st diclofenac sodium 100 mg suppository or placebo was inserted by midwife when suturing was completed and the second (50 mg diclofenac sodium or placebo) 12 hours after birth. The women involved in the study were blinded to the allocated treatment groups. Data collection sheets were completed prior to discharge from the hospital at 24 hours after birth by recall, pain score (using ten-centimetre visual analogue scale) associated with

resting, walking, sitting, and squatting. The patients who complained more pain were prescribed other analgesics (Paracetamol). Ethical approval was obtained from the research and ethical committee of the Colombo South Teaching Hospital. Informed consent has taken from all the participants. Those who did not consented were given due medical care without any discrimination.

Data were entered into a data collection sheet in the ward and confidentially stored in an ongoing electronic database. Statistical test of significance was done to analyze the result with the help of a standard statistical package, SPSS 2006. Outcome analysis was done by intention to treat with the use of parametric test (t test) and chi-square test.

Trial flowchart



During this study period, a total of 532 women gave vaginal birth at the professorial obstetric unit, Colombo South Teaching Hospital (CSTH) and sustained perineal trauma requiring suturing during child birth. A total of 235 potentially eligible women were approached in the antenatal period for participation in the trial, and 201 (85%) women provided provisional informed consent, of those women 169 became eligible after birth. All 169 women were included in the analysis; with 84 randomized to receive diclofenac sodium suppositories and 85 women received the placebo. The two groups were well balanced

for demographic characteristics at the trial entry and labour and birth outcomes as well as perineal repair technique. Study outcome data were available from 98% of women at 24 hours.

Table 1: Basic characteristics of subjects

Variables	Diclofenac Group (n=84)	Placebo Group (n=85)
Age	28.2 (yrs)	27.9 (yrs)
Gestational age	39w+ 0d	38wks+2d
Parity 1	35	33
Parity > 1	49	52
Birth Weight	2.81(kg)	2.94(kg)
Induction	31	43
Augmentation	53	42
Duration of labour	4 hrs & 50 min	4 hrs&53 min
MOD ;		
Spontaneous vaginal delivery	72	74
Instrumental Delivery	12	11

Table 2: Mean rating of pain intensity with different activities

Variables (Pain Rating)	Treatment Group : Mean (SD)		
	Diclofenac	Placebo	P Value
Sitting	2.5 (1.3)	5.0 (2.1)	<0.001
Walking	2.5 (1.1)	4.5 (1.5)	<0.001
Resting	1.7 (0.9)	3.6 (1.3)	<0.001
Squatting	2.6 (1.2)	4.6 (1.4)	<0.001
Overall	2.1(1.0)	4.0 (1.2)	<0.001

Table 3: Occurrence of significant side effects (Nausea, vomiting, gastric irritation, sensitivity)

Side Effects	Placebo	Diclofenac	Total
Present	11 (12.9 %)	14 (16.7 %)	25 (14.8%)
Not Present	74 (87.1 %)	70 (83.3 %)	144 (85.2 %)
Total	85	84	169

X^2 0.490

Table 4: Mothers' satisfaction of treatment

	More satisfied	Satisfied	Not Satisfies	Total
Placebo	10 (11.5 %)	21 (22.1 %)	54 (66.4%)	85 (100 %)
Drug	51 (63.2 %)	24 (26.1 %)	9 (11.7%)	84 (100 %)
Total	61	45	63	169

X^2 0.001

Table 2, shows mean rating of pain intensity with different activities and there is a statistically significant difference in less pain experienced by diclofenac suppository group compared to placebo group.

According to table 3, there is no statistically significant difference in occurrence of side effects in treatment group comparing to placebo group.

Table 4 demonstrates maternal satisfaction was more with diclofenac suppository comparing to placebo.

Discussion

It clearly showed from this study, use of diclofenac sodium suppository was effective safe and well accepted by mothers for perineal pain relief following childbirth.

The women who were prescribed diclofenac suppositories were more comfortable and experienced less pain. They required less additional analgesia than placebo group. While current study did not detect the

occurrence of any serious side effects associated with diclofenac administration, but still care should be maintained in prescribing this medication.

In determining the acceptability of rectal analgesic suppositories, Caroll *et al.*, interviewed 400 surgical patients, who were asked to choose between and intramuscular route of pain relief and rectal suppositories [17]. Given a choice, 18% of patients choose rectal suppositories as an acceptable method of pain relief. This current study assessed women's satisfaction of rectal route for the postnatal analgesics administration. Women who received diclofenac suppositories in our studies were more satisfied with pain relief, and overall women who took part in the study had a high degree of acceptance for the rectal route of administration of analgesia.

The half life of diclofenac sodium in plasma is one to two hours after oral administration. After rectal administration, absorption is complete in less than 40 minutes. While the half life is longer after rectal administration, the total area under the curve is similar for both preparations. Diclofenac sodium is almost completely protein bound, and as a result minimum amount of the drug is excreted in the breast milk – an important consideration for women who are breastfeeding. While rectal suppositories may be effective in reducing pain experienced after childbirth, drug effectiveness becomes a secondary consideration, if women express reluctance to the rectal route of administration. There appears to be clear advantages in using diclofenac sodium suppositories to provide short team pain relief for perineal pain after childbirth.

The strengths of our study include use of randomised blind design, several measurements of pain insensitivity of different activities and assessment of safely of NSAIDS in breast feeding mothers. The study was limited by its small sample size due to restricted duration of research.

Conclusion

The use of rectal diclofenac sodium is effective, safe and satisfactory method of analgesic for the relieving pain experienced by women following perineal trauma within the first 24 hours after childbirth.

Acknowledgements

I sincerely pay my gratitude to Dr. Rukshan Fernandopulle who directed me to make this endeavour successful and wish to thank Dr. Ananda Wijesiri (Department of Community Medicine, University of Ruhuna) for his invaluable help in data Management and statistical analysis.

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Vitamin D therapy on diabetic nephropathy

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ABSTRACT

Diabetic nephropathy (DN) is the leading cause of end stage renal disease and despite optimum therapy including ACEI/ARBs, a sizable proportion of patients with proteinuria progress to renal failure. It is likely that high renin level induced by RAS (Renin Angiotensin System) blockage may contribute to this and vitamin D is found to have an inhibitory effect over RAS as it reduces renin synthesis. This study was conducted to examine the effects of vitamin D therapy on renal functions of patients with DN.

The aims of the study were to determine the prevalence and associated factors of DN among adult diabetics attending medical clinics in Teaching Hospital, Galle (THG) and to determine the effect of vitamin D therapy on DN, cardiovascular morbidity and bone mineral density (BMD). Phase 1: Cross-sectional study involving patients with diabetes attending medical clinics in the THG. Their serum creatinine and urinary albumin (UA) levels were checked. Phase 2: A double-blind, randomized, placebo controlled study to determine the therapeutic efficacy of vitamin D. Patients with DN (UA >30 mg/g of creatinine) whose estimated glomerular filtration rate(eGFR) was more than 30 mL/min were selected and their plasma renin, Parathyroid hormone (PTH), serum vitamin D, serum calcium, serum creatinine, fasting blood sugar (FBS), lipid profile, ECG and bone mineral density (BMD) were done as baseline measurements. Subjects were randomized into two groups and treatment group was given vitamin D_3, 50000 IU (0.25ml) intramuscularly (IM) monthly for 6 months. The control group received same volume of distilled water IM. The investigations were repeated after 6 months of therapy. BMD was measured at 12months in a randomly selected subgroup of patients. The mean (SD) age was 61 (11) years and 75 % of them were females. Among them 66% had albuminuria (microalbuminuria-60.9%; macroalbuminuria-5.1%). The risk factors for albuminuria were poor glycaemic control and duration of the disease.

Prevalence of low eGFR was 42.9% (n=174); and it was associated with age and smoking. Retinopathy and neuropathy were associated with albuminuria but not with low eGFR.

Of 155 patients invited, 85 were randomly assigned to two groups after exclusions and 82 completed the study. After six months, mean reduction of urinary albumin to creatinine ratio in the treatment and control group were 51.8 mg/g (P=0.06) and 22.4 mg/g (P<0.001), respectively (between group difference P=0.001). Significant increase in the eGFR was observed in the treatment group while eGFR remained unchanged in the control group (P=0.006 for the between-groups difference). Mean reduction in plasma renin in the treatment group and control group were 5.85 pg/mL (P < 0.001) and 0.95 pg/mL (NS), respectively. After vitamin D treatment, total body BMD, total body BMC (bone mineral content) and BMDs of total spine, femoral neck and total hip regions increased by 2.0%, 2.2%, and 1.8%, 2.1% and 2.6% (P<0.05 for all), respectively in the treatment group. In the same group after 6 months of stopping treatment, marginal but a statistically significant reduction of total BMD and BMC was observed (P=0.009) while all regional BMDs remained unchanged. In the control group, none of the BMD/BMC measurements changed significantly during trial and post-trial period. No significant effect was observed in cardiovascular risk scores. In conclusion, vitamin D_3 has beneficial effects on patients with diabetic nephropathy.

This study was performed in University of Ruhuna, Sri Lanka and the results were included in a thesis with two published papers for a PhD degree with the University of Ruhuna, Sri Lanka and defended the thesis on 19th of February 2015.

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Introduction

Sri Lanka, being a developing country with a population of 20.7 million, is experiencing an epidemic of diabetes mellitus [1]. The burden of diabetic nephropathy should be taken into consideration both in estimating health care cost and in planning health care services in the country.

Although Sri Lanka has a high prevalence of diabetes, prevalence of nephropathy and factors that are associated with nephropathy are not well known. Methodological differences such as definition of nephropathy and characteristics of study subjects have lead to a wide variation in estimations. Prevalence of nephropathy among patients with diabetes in Sri Lanka varies from 20% to 33% [2, 3]. Most of these studies have been conducted in diabetes clinics in Colombo and defining diabetic nephropathy in some analyses is somewhat doubtful. Many studies have used single spot urine sample to detect microalbminuria and also different cut-off values such as 50 mg/L and 20 g/dL[2, 3, 4].

Microalbuminuria is defined as excretion of 30 - 300 mg of albumin in 24 hr urine collections, 20-200 µg/min in timed urine collection, or 30 - 300 mg/g of creatinine in spot urine collections, on two of three urine collections [5]. Previous studies conducted in Sri Lanka did not follow this standard definition in determining microalbuminuria. Hence phase 1 of this study was carried out to determine the prevalence of nephropathy among patients with diabetes according to the standard methods of defining nephropathy in patients with diabetes in Teaching Hospital Galle (THG).

Diabetic nephropathy is the commonest cause of end stage renal disease worldwide [6, 7]. The current management strategies of this condition target the Renin Angiotensin Aldesteron System (RAAS) as derangements in the RAAS are suspected to play a critical role in the aetiology or progression of diabetic nephropathy [8, 9]. Inhibitors of RAAS namely Angiotensin Converting Enzyme Inhibitors (ACEI) and Angiotensin Receptor Blockers (ARB) are included in the standard treatment for reducing proteinuria. Despite the optimum use of these agents, renal disease in some tends to progress and this has been attributed to the residual proteinuria not reversed by the above agents [10, 11].

Current data on the role of vitamin D reducing the progression of renal damage in diabetic renal disease are limited and come from either animal studies or few human studies [12]. Studies which explored the effect of vitamin D on animal models of kidney disease have reported the beneficial effects of such therapy. Li *et al.* demonstrated that 1, 25 dihydroxy vitamin D3 (1, 25(OH) $_2D_3$), the active form of vitamin D, has an inhibitory effect over RAAS as it reduces renin synthesis, the first and the rate limiting step in the RAAS [13]. In normal mice, vitamin D deficiency stimulated renin expression whereas the treatment with vitamin D reduced the synthesis of rennin [13] . Further, Zhongyi *et al.* reported that the combined treatment with losartan and vitamin D analogue completely eliminate the albuminuria of diabetic mice [14].

Randomized control trials examining the effect of vitamin D on the progression of proteinuria are limited[15],[16]. Further, these studies are not sufficiently powered to generate conclusive results. There is a paucity of sufficiently powered randomized controlled trials examining the different reno-protective effects of vitamin D among patients with diabetic nephropathy.

Further, Vitamin D supplementation is an emerging potential approach to reduce burden of CVD (coronary vascular disease) in diabetic nephropathy through its favourable effects on insulin resistance and the cardiovascular risk profile. Most of the previous studies on vitamin D and CVD have used conventional doses of vitamin D and not conducted as placebo controlled trials[12, 15]. Since the pleotropic effects of vitamin D are reported with higher doses, such doses should be used to determine the effects of vitamin D on cardiovascular risk factor profile in patients with early stages of diabetic nephropathy.

Diabetes which is associated with many chronic, metabolic abnormalities could adversely affect the bone strength. Both type 1 and type 2 diabetes are associated with an increased risk of fragility fractures and osteoporosis [17]; [18]. Vitamin D has a well-established role in bone health. No studies have examined the effect of vitamin D on BMD/BMC among patients with early diabetic nephropathy. Osteoporosis and diabetes are prevalent diseases in Sri Lanka and the number of patients with this disease combination can

be expected to increase in the future [19]. Therefore, it is important to examine the effects of vitamin D_3 treatment on BMD in patients with diabetes with mild renal insufficiency.

Hence phase 2 of this study was done to evaluate the effect of vitamin D therapy in reversing the progression of diabetic nephropathy and also the effect on plasma renin, CVD risk profile, BMD and BMC measurements.

Materials and methods

Phase 1

Cross-sectional study included randomly selected patients with diabetes attending medical clinics at Teaching Hospital Galle. Diagnosis of microalbuminuria or overt nephropathy was made if urinary albumin excretion was between 30 – 299 mg/g of creatinine and >300 mg/g of creatinine, respectively. We repeated the urine test in positive cases at an interval of two weeks and if the second sample was found negative a third sample was also tested. They were labelled positive only when two samples gave positive results. Ongoing urinary tract infection was excluded by urine strip method in subjects with albuminuria. Demographic data, and presence of other macrovascular and microvascular changes were also noted in all patients. An estimated GFR (eGFR) <60 mL/min was taken as the cut-off for defining low-eGFR.

Phase 2

Patients with early diabetic nephropathy (urinary albumin >30mg/g of creatinine and eGFR more than 30 mL/min) were recruited from phase 1 of the study. Other causes of albuminuria were excluded before urine analysis for microalbuminuria and repeated measurements were done to confirm the findings. Those with uncontrolled blood pressure (>130/80 mmHg over the last two clinic visits), hyperphosphataemia (Serum phosphate > 5mg/dL), hypercalcaemia (Serum total Ca>10 mg/dL), uncontrolled blood sugar (HbA1c>8%) chronic liver disease, hyperthyroidism, hyperparathyroidism, decompensated heart failure or diseases related to calcium or vitamin D metabolism were excluded. Other causes of proteinuria such as

current urinary tract infection, urolithiasis, and renal tuberculosis were excluded by history, examination and relevant investigations.

Study design

Patients were allocated to two groups by Block randomization method (block of 2) using a random number table. Concealed envelopes containing treatment allocation were given to research assistants who assigned participants to treatment and control groups. Treatment group received monthly dose of 50,000 IU of vitamin D3 intramuscularly and the control group was given an equal volume of distilled water (0.25 mL) to the same site in a similar manner. Participants, those administering the interventions, clinicians, and those assessing the outcomes were blinded to the group assignment.

Study Procedures

Patients underwent a detailed medical history, a physical examination including systolic and diastolic blood pressure (SBP and DBP) measurement. Blood and urine were collected for the baseline measurements which included serum creatinine, serum calcium, urine microalbumin, fasting glucose (FBS), serum calcium, phosphate, creatinine, Parathyroid Hormone (PTH), renin and vitamin D level and lipids namely total cholesterol (TC), low density lipoprotein (LDL), triglycerides (TG), and high density lipoprotein (HDL).

A safety visit was scheduled one week after starting the trial to monitor calcium and phosphorus concentrations and to elicit any adverse events. The protocol specified withdrawal from the trial if serum calcium exceeded 11 mg/dL.

All patients underwent whole body dual-energy X-ray absorptiometry (DXA) scan and BMD and BMC of the total body, total spine (L_1-L_4) and proximal femur were measured. All scans were performed and analysed by the same technician adhering to the manufacturer's protocol. DXA machine was calibrated using the calibration phantom provided by the manufacturer. The precision error of the machine has been published previously [20]. There were no software or hardware changes during the study period.

After six months of treatment all the measurements done at the baseline including DXA were repeated. When the trial period of six months was over, a randomly selected subgroup of patients (25 from each group) was followed up for further six months and another DXA testing was performed.

Biochemical assays were performed using commercial kits. Intact PTH (Immunotech, IRMA PTH), renin (Beckman coulter, IRMA Active Renin) by radioimmunoassay and 25-hydroxy vitamin D were measured using immunochemiluminometric (Vitros immunodiagnostic) assays. Serum creatinine was measured by spectrophotometric method with an alkaline-pictrate solution.

Ethical aspect

Ethical clearance was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka. Clinical trial has been registered in the local clinical trial registry. Informed written consent was obtained from all subjects and the study.

Statistical analysis

The baseline characteristics between the two groups were compared by either unpaired t-test or Chi-square test. Changes in urinary albumin, renal functions, vitamin D, renin, PTH, BMD/BMC during the trial period were analysed by the Repeated measure ANOVA (SPSS, Chicago, USA). P value was adjusted for multiple comparisons by the Bonferroni method.

Results

Phase 1

The mean (SD) age of the total study sample (n=480) was 61 (11) years and 75 % of them (n=360) were females. Of the diabetic subjects studied, 286 (60.9%) had microalbuminuria and 24 (5.1%) had macroalbuminuria. Since the number of patients with macroalbuminuria was small we considered both micro and macroalbuminuic patients together in the rest of the analyses. Hence the prevalence of diabetic nephropathy defined according to albuminuria was 66%.

Patients with albuminuria had a longer duration of diabetes when compared with normoalbuminuric patients ($P<0.05$). Systolic blood pressure, age and poor glycaemic control were significantly higher among patients with albuminuria when compared with normoalbuminuric patients ($P < 0.05$). Smoking, BMI (body mass index), DBP and gender and WHR (waist hip ratio), were not significantly different between patients with albuminuria and normoalbuminuria.

Regression analysis revealed that glycaemic control and duration of diabetes were significant associations of albuminuria in patients with diabetes. Although there were many variables associated with albuminuria, the regression model retained only poor glycaemic control and disease duration as significant associations of albuminuria.

When, nephropathy was defined based on the eGFR regardless of albuminuria, according to the regression analysis, smoking and age were the significant determinants of low eGFR.

One hundred and seventy four (42.9%) patients had low eGFR and 43 (43/174, 24.7%) of them had normoalbuminuria. In the total sample, the proportion of patients with low eGFR and normoalbuminuria was 43 (10.8 %).

When other microvascular complications were considered, retinopathy and neuropathy were associated with albuminuria but not with the low eGFR.

Phase 2

A total of 157 patients were invited for the study and 72 were excluded due to the presence of one or more exclusion criteria. Remaining 85 were randomly assigned to two groups and 82 subjects completed the study; Forty-one patients from each group completed the study. Data were analysed by the intention-to-treat method.

No significant differences were found with regards to the baseline characteristics between the treatment and control groups. All patients received either an ARB or ACEI at the baseline. During the study period,

oral hypoglycaemic drugs were increased in nine patients (six in the treatment group). Losartan was increased in three patients (two in the control group).

Table 1 shows the changes of the urine microalbumin to creatinine ratio, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood sugar (FBS), serum creatinine, eGFR, PTH, renin and vitamin D levels after three and six months of treatment in the treatment and control groups. After six months, mean reduction of urinary albumin to creatinine ratio was 51.8 mg/g ($P \leq 0.001$) in the treatment group, 22.4 mg/g ($P = 0.06$) in the control group and this difference was significant ($P = 0.001$). Significant increase in the GFR was observed in the treatment group while in the control group GFR remained unchanged ($P = 0.006$ for the between-groups difference). There was a significant reduction of serum creatinine in the treatment group but not in the control group. But the change was not significant between groups.

A significant increase of SBP was seen in the control group whereas SBP remained unchanged in the treatment group and the difference was not statistically significant. Significant trends in the DBP was seen in both groups during the study period but the difference between the two groups was not statistically significant ($P = 0.17$). Significant reduction of FBS was seen only in the control group and the difference between groups was not statistically significant ($P = 0.23$).

Significant reduction of PTH was observed in both treatment and the control groups, but the change between two groups was not statistically significant ($P = 0.26$). In the treatment group, vitamin D level increased by 25.64 nmol/L and between the two groups the change was statistically significant ($P < 0.001$). Mean reduction in plasma renin in the treatment group was 5.85 pg/mL ($P < 0.001$). In the control group the reduction observed was only 0.95 pg/mL. The difference between the two groups was statistically significant ($P = 0.006$) (Table 1).

A significant inverse correlation was observed in vitamin D with percentage change in plasma renin level (ρ=-0.66, $P < 0.01$) and percentage change in urine albumin levels (r=-0.47, $P < 0.01$). Furthermore,

percentage changes of renin and urinary albumin also showed a significant correlation ($\rho = 0.62$, $P < 0.01$) (Table 2).

Vitamin D therapy significantly reduced DBP, total cholesterol and LDLC but the between group differences were not significant (Table 3). There was an increase in HDL cholesterol level in the treatment group while there was no change in the control group (the between groups difference was significant)

Table 4 shows the changes of the total body BMD/BMC, regional BMDs, total fat and lean masses, during the initial 6 months of treatment in the treatment and control groups.

After six months of vitamin D injections, in the treatment group total body BMD, total body BMC and BMDs of spine, femoral neck and total hip regions increased by 2.0%, 2.2%, 1.8%, 2.1% and 2.6% ($P<0.05$ for all), respectively (Table 5). Increase observed in the BMD measurement in the trochanteric region was not statistically significant among the patients in the treatment group.

In the control group, total body BMC, BMD, or regional BMDs did not change significantly during the initial 6 months. Furthermore, there was no significant difference in either total fat or lean mass in any of the groups before and after treatment.

Table 6 shows the changes observed in the BMD measurements at six months after stopping treatment in subgroup of patients who had undergone the 3rd DXA scan.

After 6 months of stopping treatment, a statistically significant reduction of total BMD and BMC was observed in the treatment group ($P=0.009$). In the same group, changes in the regional BMDs were not statistically significant.

In the control group none of the BMD/BMC measurements changed significantly during the post-trial follow up 6 months period.

No adverse events, particularly hypercalcaemia were reported during the study period.

Discussion

The cross-sectional study involving patients with diabetes attending medical clinics, Teaching Hospital Galle, showed the prevalence of diabetic albuminuria to be 66% (95% CI: 61.7 to 70.2). When defined by the degree of albuminuria, the prevalence of microalbuminuria was 60.9% (95% CI: 56.5 to 65.3) while macroalbuminuria prevalence was 5.1% (95% CI: 3.1 to 7.1). The risk factors for albuminuria included poor glycaemic control and the duration of the disease.

Among study subjects, the prevalence of low eGFR was 42.9% (95% CI: 38.1 to 47.7) and age and smoking were the factors related to the low eGFR. Other microvascular complications namely, retinopathy and neuropathy were associated with albuminuria but not with low eGFR.

We found different determinants or associations for albuminuria and eGFR among these adult diabetics. This discrepancy could, partly be due to the fact that these two processes are independent with own risk factors and associations. Another reason could be that the eGFR may not be an accurate reflection of renal functions of these patients since eGFR in these patients were calculated using a formula developed for European patients. Acute or chronic infections too can cause albuminuria without renal insufficiency. The research protocol required to inquire and examine all subjects for the presence of either acute or chronic ongoing urinary infections.

A sizable proportion of patients with low eGFR had normal albumin excretion (normoalbuminuric renal insufficiency). Among patients with renal insufficiency 26.7% were normoalbuminuric. The majority of patients with normoalbuminuric renal insufficiency did not have either retinopathy or neuropathy suggesting that the aetiology of renal insufficiency could be related to causes other than diabetes in these patients.

Results from the Phase 1 of the study indicate the magnitude of the growing epidemic of diabetic nephropathy in the local set up. It can be recommended that all diabetics be screened to assess their renal function by both albuminuria and by eGFR.

The randomized, double-blind placebo controlled clinical trial conducted among patients with diabetic nephropathy showed a significant reduction of urine microalbumin, serum creatinine and improvement of GFR after monthly injection of vitamin D for six months. These results are supportive of the reno-protective effects of high dose vitamin D in diabetic patients with nephropathy who are on optimum medical therapy. Furthermore, we observed a significant reduction of renin levels in the treatment group compared to the control group.

Vitamin D increased BMD/BMC compared to placebo. This improvement was observed in the total body BMD/BMC and BMDs of total hip, total spine and femoral neck. The regional BMDs remained unchanged 6 months after withdrawing vitamin D treatment while only a marginal loss was observed in total BMD and BMC. Vitamin D caused no significant effect on cardiovascular risk scores, blood pressure or major serum lipid components.

The dose of vitamin D used in this study raised serum vitamin D level substantially. Although this was sufficient to demonstrate reno-protective effect and benefit on BMDs the period of trail was insufficient to demonstrate a positive effect on cardiovascular measurements.

Based on the results of this study, vitamin D can be considered as an add-on therapy to patients with increasing microalbuminuria despite optimum glycaemic and blood pressure control and receiving maximum tolerable doses of ACEI or ARB.

Due to the paucity of data, however, further clinical trials should be done to reproduce the results observed in this study. If the same benefits are proven, use of vitamin D for complete suppression of albuminuria can be recommended.

Cardiovascular benefits of high dose of vitamin D cannot be ruled out, completely, based on the data observed in this study. Since patients recruited had good blood pressure and glycaemic control, further reductions of these measurements were not possible. Further studies on patients with uncontrolled blood pressure and poor glycaemic control may reveal these benefits.

BMD/BMC increase is an important finding so that the treatment of vitamin D in early diabetic nephropathy will delay the occurrence of renal bone disease. But further clinical trials are needed to test this hypothesis in patients with diabetes.

Conclusions

The prevalence of albuminuria was 66% (95% CI: 61.7 to 70.2) in patients with diabetes who were attending medical clinics, Teaching Hospital Galle. The risk factors for albuminuria included poor glycaemic control and the duration of the disease.

The prevalence of low eGFR was 42.9% (95% CI: 38.1 to 47.7) and age and smoking were the factors related to low eGFR. Other microvascular complication namely, retinopathy and neuropathy were associated with albuminuria but not with low eGFR.

Monthly injections of high dose vitamin D3 has improved the renal functions in patients with diabetic nephropathy, BMD and BMC. This treatment did not have a significant effect on cardiovascular risk scores or blood pressure. Further studies involving longer durations of treatment at different doses of vitamin D may be needed to reconfirm these findings.

Table 1: Changes observe in the treatment and control groups at 3 months and 6 months

Variable		Baseline	At 3 months	At 6 months	P within group	P between group
SBP (mmHg)	Control	121 (7)	121 (8)	127 (6)	< 0.001	0.07
	Treatment	120 (8)	120 (8)	121 (7)	0.59	
DBP (mmHg)	Control	70 (6)	72 (6)	72 (6)	< 0.001	0.17
	Treatment	71 (6)	69 (6)	68 (6)	< 0.001	
FBS (mg/dL)	Control	130.2 (12.5)	130.6 (10.1)	127.8 (10.7)	0.02	0.23
	Treatment	128.3 (13.6)	125.8 (13.4)	125.9 (10.9)	0.08	
PTH (pg/mL)	Control	42.5 (19.0)		37.6 (12.6)	0.003	0.26
	Treatment	38.2 (11.3)		35.7 (7.9)	0.001	
25 (OH)D (nmol/L)	Control	49.64 (16.46)		45.67 (17.20)	0.004	< 0.001
	Treatment	56.11 (12.95)		81.75 (15.03)	< 0.001	
Plasma renin (pg/mL)	Control	15.14 (4.82)		14.19 (4.6)	0.02	0.006
	Treatment	14.64 (5.62)		8.83 (4.81)	< 0.001	
Urine albumin (mg/g)	Control	185.8 (50.6)	160.9 (63.4)	163.4 (56.2)	0.06	0.001
	Treatment	169.4 (35.8)	122.1 (54.4)	117.6 (45.2)	< 0.001	
Serumcreatinine (mg/dL)	Control	0.87 (0.22)	0.87 (0.20)	0.87 (0.20)	0.84	0.10
	Treatment	0.86 (0.13)	0.80 (0.12)	0.77 (0.11)	< 0.001	
GFR (mL/min)	Control	68.7(20.3)	68.2 (19.3)	68.8 (20.5)	0.81	0.006
	Treatment	77.2 (20.9)	85.8 (26.3)	83.1 (24.4)	< 0.001	

SBP (systolic blood pressure), DBP (diastolic blood pressure), PTH (parathyroid hormone), FBS (fasting blood sugar), GFR (glomerular filtration rate)

Table 2: Correlations (Spearman rho) between the percentage change in vitamin D, urine albumin, plasma renin and PTH

Percentage change	Urine albumin	Renin	PTH
Vitamin D	-0.47**	-0.66**	-0.02
Urine albumin		0.62**	-0.08
Renin			-0.02

**Correlations are significant at 0.01 level

Table 3: Changes in CVDR factors and risk scores in the test and control groups

Variable		Baseline	At 3 months	After 6 months	P value within group	P value between group
SBP	Control	121 (7)	121 (8)	127 (6)	< 0.001	
(mmHg)	Test	120 (8)	120 (8)	121 (7)	0.59	0.07
DBP	Control	70 (6)	72 (6)	72 (6)	< 0.001	
(mmHg)	Test	71 (6)	69 (6)	68 (6)	< 0.001	0.17
TC (mg/dL)	Control	194.6 (32.1)	193.6 (30.8)	196.9 (31.4)	0.24	
	Test	194.8 (30.1)	191.5 (28.1)	185.7 (27.2)	< 0.001	0.50
TG (mg/dL)	Control	128.4 (50.8)	127.9 (49.5)	128.7 (45.3)	0.62	
	Test	122.8 (41.4)	121.8 (40.1)	118.2 (32.4)	0.062	0.44
LDL	Control	117.0 (28.1)	114.6 (28.9)	117.1 (30.2)	0.34	
(mg/dL)	Test	119.7 (28.7)	115.7 (27.6)	106.10(26.5)	< 0.001	0.7
HDL	Control	53.5 (10.9)	53.7 (10.7)	53.9 (9.7)	0.40	
(mg/dL)	Test	50.3 (7.5)	51.5 (7.1)	55.7 (6.8)	< 0.001	< 0.001

SBP (systolic blood pressure), DBP (diastolic blood pressure), TC (total cholesterol), TG (triglyceride), LDL (low density lipoprotein), HDL (high density lipoprotein)

Table 4: Changes bone mineral density and fat mass in the treatment and control groups.

Variable		Baseline	After 6 months	Percentage difference	P within groups	P between groups
BMD	Control	1.038 (0.121)	1.031 (0.191)	-0.67	0.75	0.61
	Treatment	1.038 (0.120)	1.059 (0.107)	2.02	0.01	
BMC	Control	1775.63 (412.76)	1721.64 (369.70)	-3.04	0.074	0.73
	Treatment	1757.95 (383.68)	1795.85 (373.27)	2.16	0.007	
Spine BMD	Control	0.848 (0.132)	0.836 (0.119)	-1.41	0.27	0.72
	Treatment	0.845 (0.153)	0.860 (0.142)	1.78	0.04	
Femoral neck BMD	Control	0.722 (0.109)	0.712 (0.094)	-1.38	0.23	0.43
	Treatment	0.731 (0.153)	0.746 (0.142)	2.05	0.03	
Trochanter BMD	Control	0.607 (0.089)	0.604 (0.08)	-0.49	0.5	0.46
	Treatment	0.615 (0.111)	0.627 (0.103)	1.95	0.07	
Hip BMD	Control	0.857 (0.113)	0.852 (0.105)	-0.58	0.56	0.25
	Treatment	0.876 (0.148)	0.899 (0.149)	2.62	0.008	
Total fat mass	Control	15.85 (6.67)	16.48 (6.16)	3.99	0.20	0.2
	Treatment	17.41 (5367.460)	18.21 (5.56)	4.6	0.06	
Lean mass	Control	37.04 (6.94)	36.98 (6.38)	-0.18	0.86	0.16
	Treatment	38.92 (8.32)	39.64 (7.73)	1.85	0.09	

Table 5: Percentage changes in BMDs, fat mass and lean mass in different regions in the control and the treatment groups

	BMD	BMC	Spine BMD	Femoral neck	Trochanter BMD	Hip BMD	Total fat mass	Lean mass
Control	-0.67	-3.04	-1.41	-1.38	-0.49	-0.58	3.99	-0.18
Treatment	2.02	2.16	1.78	2.05	1.95	2.62	4.6	1.85

Table 6: Changes bone mineral density and fat mass in the treatment and control groups

Variable		After 6 months	After 12 months	Percentage difference	P within groups	P between groups
BMD	Control	0.999 (0.134)	1.006 (0.112)	0.70	0.47	0.21
	Treatment	1.054 (0.120)	1.041 (0.131)	-1.23	0.009	
BMC	Control	1735.92 (430.22)	1716.05 (402.87)	-1.14	0.26	0.54
	Treatment	1808.19 (450.57)	1795.94 (458.27)	-0.68	0.04	
Spine BMD	Control	0.823 (0.128)	0.828 (0.126)	0.61	0.19	0.69
	Treatment	0.847 (0.168)	0.837 (0.163)	-1.18	0.07	
Femoral neck BMD	Control	0.711 (0.109)	0.711 (0.105)	0	0.92	0.28
	Treatment	0.756 (0.166)	0.753 (0.169)	-0.4	0.48	
Trochanter BMD	Control	0.601 (0.823)	0.598 (0.851)	-0.5	0.35	0.55
	Treatment	0.617 (0.112)	0.615 (0.110)	-0.32	0.33	
Hip BMD	Control	0.851 (0.114)	0.848 (0.116)	-0.35	0.69	0.3
	Treatment	0.889 (0.160)	0.895 (0.160)	0.67	0.48	
Total fat mass	Control	16.10 (6.75)	15.66 (6.57)	-2.71	0.06	0.22
	Treatment	18.28 (5.31)	17.98 (5.20)	-1.61	0.79	
Lean mass	Control	37.39 (7.36)	37.29 (7.16)	-0.26	0.52	0.2
	Treatment	40.25 (8.54)	40.35 (8.65)	0.23	0.54	

BMD (bone mineral density), BMC (bone mineral content

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Effect of iodine and iron status during pregnancy on maternal and neonatal thyroid functions: A prospective cohort study in Bope - Poddala health division

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ABSTRACT

Iodine and iron are the most common nutritional deficiencies in the world. It was reported that even mild iodine deficiency during pregnancy will affect maternal and subsequently neonatal thyroid functions and iron deficiency has multiple adverse effects on thyroid metabolism. Since iodine and iron deficiencies are common in pregnant women in Sri Lanka, its ultimate effect will be on the new born. The aim of the study was to assess the iodine and iron status in women during pregnancy and its effects on thyroid function of the mother and the newborn.

The study was carried out in the Bope-Poddala MOH division of Galle District. Four hundred and twenty-five pregnant women were enrolled and they were followed up during the course of the pregnancy until delivery. Maternal iodine and iron status was assessed using different parameters and its effect on babies was assessed estimating neonatal urine iodine (UI) and neonatal thyroid stimulating hormone (nTSH).

The median maternal UI concentration of the sample was 175.2µg/L (IQR 106.3-263.4 µg/L),126.0µg/L (IQR 74.8 - 196.4µg/L),106.0µg/L (IQR 67.4-160.6 µg/L) in the first, second and third trimesters respectively indicating progressive reduction with the advancement of the pregnancy ($p = <0.001$). 41.7%mothers had insufficient UI concentration at the study entry and it was increased to 58.8% and 72.9% in the 2nd and 3rd

trimesters. Median serum TSH in the 1st trimester, 1.3 mIU/mL (IQR 0.8 – 1.8 mIU/mL) was significantly increased ($p<0.001$) to 1.6 mIU/mL (IQR 1.2 – 2.1 mIU/mL) at the 3rd trimester. Median vales of fT_4 for 1st and 3rd trimesters were 18.0 pmol/L and 15.5 pmol/L ($p=0.002$) respectively. Results confirmed poor iodine nutrition by UI during pregnancy and role of iodized salt in maintaining iodine nutrition throughout pregnancy was questionable. In contrast maternal thyroid status was maintained within reference range. Regarding salt iodine content > 50% of brand did not contain iodine within recommended range and this may be a contributing factor to the poor iodine nutrition seen among pregnant women. Only 10.9% of neonates had insufficient UI level (< 100µg/L) and the median (IQR) UIC level was 105.20 (81.25; 142.00) µg/L indicating sufficient UI level. The median neonatal TSH level was 3.55 (2.50; 6.50) mIU/mL whereas 37.7% of neonates had neonatal TSH>5.0 mIU/mL indicating moderate iodine deficiency according to WHO criteria. Neonatal UI level had significant positive correlations with maternal 3rd trimester UI ($r=0.23$; $p<0.001$) but such a significant correlation was not observed between maternal UI and neonatal TSH. Prevalence of anaemia was low in early pregnancy (4.8%) but iron deficiency was significantly high (42.1% had ferritin < 15ng/mL). Iron status was significantly improved at the end of the pregnancy most probably due to iron supplements. It was observed that maternal iron status had no significant effect on maternal as well as neonatal thyroid functions in this sample.

Although neonatal thyroid status was normal according to current reference values, it is worthwhile to assess long term effects of inadequate iodine status of mothers on the offspring. Iodine content of the salt products must be tightly regulated and manufacturing should be closely monitored.

This study was performed at the Department of Biochemistry and Nuclear Medicine Unit, Faculty of Medicine, University of Ruhuna, Sri Lanka. The results were published in three original papers in peer reviewed journals. In addition, seven abstracts were presented in national and international forums. The thesis was defended on 17th November 2015.

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Introduction

Iodine is a vital micronutrient required at all stages of life; foetal life and early childhood being the most critical phases of requirement [1]. Iodine deficiency during pregnancy and infancy may impair growth and neurodevelopment of the offspring and increase infant mortality [2]. Diet is the sole source of iodine, and it depends upon the iodine content of water and soil [3]. In many countries, iodized salt is the major source of iodine.

Pregnancy has a profound impact on the thyroid gland and thyroid function and it is a stress for the thyroid gland. Production of thyroxine (T_4) and triiodothyronine (T_3) increases by 50%, along with a 50% increase in the daily iodine requirement [4].

Worldwide, about 39 million newborns are at risk of lowered intellectual capacity because of iodine deficiency, every year. It is important to note that maternal iodine deficiency is one of the major causes for children not to reach their full potential and on a list of modifiable biological and psychosocial risks encountered by young children; iodine deficiency was ranked in the third place [5].

The ongoing monitoring of population iodine status remains crucially important and particular attention may need to be paid to monitoring the status of vulnerable populations, such as pregnant women and infants [6]. Several indicators are used to assess the iodine status of apopulation: thyroid size by palpation and/or by ultrasonography, urinary iodine (UI) and the blood constituents, TSH and thyroglobulin [7].

According to a report published by the WHO in 2004 iodine intake in Sri Lanka was categorized as adequate and iodine nutrition as optimal [8]. It indicated that Sri Lanka has achieved a satisfactory control of iodine deficiency disorder (IDD) after the initiation of universal salt iodization programme in 1995. But mild to moderate iodine deficiency can still be there in a considerable percentage of population which was not screened.

However, a study done in Sri Lanka very recently revealed that median urinary iodine level in pregnant women was 113.7 µg/L, which is far below the WHO recommendation (between 150 and 249 µg/L), indicating inadequate iodine status of pregnant women in Sri Lanka and it was also

found in 2010 that only 69.4% of salt at household level contained an adequate iodine concentration of >15 ppm in Sri Lanka [9]. These findings raised a question about the iodine nutrition status in Sri Lankan population at present especially in pregnant women, after many years of salt iodization.

Iron deficiency (ID) is the most common nutritional deficiency state in the world, affecting more than two billion people globally (10). Iron deficiency can cause several adverse effects in various stages in life. It adversely affects the cognitive performance, behaviour, and physical growth of infants, preschool and school-aged children. Iron deficiency anaemia during pregnancy increases perinatal risks for mothers and neonates and increases overall infant mortality [11].

ID has multiple adverse effects on thyroid metabolism [12, 13]. It decreases circulating thyroid hormone concentrations, likely through impairment of the haem-dependent thyroid peroxidase (TPO) enzyme [14]. It is obvious that there is an association between iron status and thyroid hormone status and since both iodine and iron deficiencies is common during pregnancy it is worthwhile to assess their effect on maternal and neonatal thyroid functions.

It has been shown that the consumption of iodized salt is not sufficient to maintain the optimal iodine level throughout the pregnancy even in some of the developed countries [15]. Therefore, it is important to assess the iodine level in each trimester to obtain the data of how universal salt iodization (USI) programme in Sri Lanka is successful in maintaining iodine nutrition throughout the pregnancy. The ultimate effect of maternal iodine and iron nutrition will be on the new born babies. Therefore, it is important to evaluate the thyroid function and iodine status of their new born babies by measuring blood spot TSH assay and urinary iodine excretion in newborn. This will finally give a clear idea of effectiveness of the iodized salt consumption in maintaining iodine nutrition during pregnancy and the effect of iron status during pregnancy on the thyroid function of the mother and the baby.

Methods

This study was conducted as a prospective cohort study in Bope- Poddala Health Division of the Galle District in the Southern Province. Required sample for this study was taken as 425 assuming that 50% of pregnant women were deficient in iodine with inflation of 10% to cover up possible termination of pregnancy during the study period and the dropouts.

All pregnant women with gestational age: ≤ 12weeks (as judged by the date of last menstrual period) who attended the five Maternal and Child Health (MCH) clinics in the Bope-Poddala MOH division were included. Pregnant women with previous history of thyroid and renal diseases were excluded.

Study was conducted in four phases. In the first phase of the study, basic details were obtained and size of the thyroid gland was assessed by palpation method and by ultra sound scanning. A spot urine sample was collected for urine iodine estimation. A blood sample was collected to assess thyroid function (thyroid stimulating hormone (TSH), free thyroxin (fT_4) and serum thyroglobulin level), serum ferritin (SF) and haemoglobin (Hb) levels. Data collection was done by using an interviewer administered questionnaire as the tool. The pregnant women recruited for the study were followed at their respective clinics throughout the pregnancy period.

In the second phase, the subjects were met in their 2nd trimester and details about iron supplementation, information regarding gestational age at the time of second visit were obtained. A spot urine sample was collected again to assess the UI level in the second trimester.

In the third phase, the study subjects were visited again in their 3rd trimester and further details about iron supplementation, expected place of delivery and any change in contact details were obtained. A spot urine sample was collected to assess the UI level in the third trimester. Further, another blood sample was collected to assess same parameters as in the first phase.

In the fourth phase, details of the newborn were recorded. A heel prick blood sample and a urine sample were collected for the analysis of serum TSH and UI concentrations respectively.

Salt samples (both table salt and crystal salt) were collected from places where salt was being sold in the study area to determine the iodine content.

Urine iodine concentration was measured using ammonium persulfate method and the iodine content of iodated salt samples was measured using the iodometric titration method recommended by WHO/UNICEF/ ICCIDD (16) at the iodine contamination free laboratory of the Department of Biochemistry, Faculty of Medicine, Galle. To measure serum fT_4, TSH and thyroglobulin immuno assay test kits (MP Biomedicals, Diagnostic Division, USA) were used. The Ferritin Immunoassay Test Kits (MP Biomedicals, Diagnostic Division, USA) were used to estimate the serum ferritin concentrations and haemoglobin estimation was done by using automated haematology analyzer (Sysmex, USA).The DELFIA neonatal TSH kits (WasllacOy, Finland) were used to estimate the neonatal TSH concentrations.

Data were analyzed using SPSS version 17.0 for Windows. Descriptive data were presented as mean and standard deviation (SD) or median and interquartile range unless stated otherwise. Correlation and regression models were used to examine the associations between urinary iodine and other variables recorded as continuous numerical variables. When comparing different groups, student t-test (for two groups), and analysis of variance (for three or more groups) was used to detect differences in numerical variables and Chi square test was used to detect differences in categorical variables. Details of the statistical calculations used for individual analysis are given in the relevant results sections. Two-tailed p value less than 0.05 was selected as the level of statistical significance.

Ethical approval for the study was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka and informed written consent was obtained from each of the participants who were selected for the study.

Results

Iodine content of iodized salt

There were 89 salt samples of salt comprising 30 of crystal and 59 of powdered salt (table salt) belonged to 42 different brands (Table 1). In the total sample, there were 15 brands of crystal salt and 27 of powdered forms.

The overall median iodide level of the total sample was 20.41 ppm (range 0.0 to 73.81) where as the crystal salt had a median of 17.77 ppm (range 3.70 to 73.81) and powdered salt had 21.15ppm (range 0.0 to 41.24). Although the median iodine level was found within the specified range (15-30 ppm) our analysis revealed that 23.6% of samples (11 crystal and 10 powdered salt), had iodide levels below 15ppm and one powdered salt sample did not even contain a detectable amount. On the other hand 12.4% samples (4 crystal and 7 powdered) had iodine level above the upper limit of the recommendation.

Table 1: Characteristics of salt usage & the type[1]

Characteristics	Salt type		Significance[2]
	Crystal (n=30)	Powder (n=59)	(C^2 - value)
Place of purchase			
Retail shops	20 (22.5)	37 (41.6)	0.52; p=0.85
Supermarkets	7 (7.9)	17 (19.1)	
Weekly fair	3 (3.4)	5 (5.6)	
Exposure to sunlight			
Yes	7 (7.9)	10 (11.2)	0.63; p=0.47
No	23 (25.8)	49 (55.1)	
Shelf life			
<365 days	22 (24.7)	53 (59.6)	4.08; p=0.04
≥365 days	8 (9.0)	6 (6.7)	
Period of storage			
<90 days	18 (20.2)	34 (38.2)	0.05; p=0.83
≥90 days	12 (13.5)	25 (28.1)	
Place of storage			
Shelf	15 (16.9)	33 (37.2)	0.28; p=60
Floor	15 (16.9)	26 (29.1)	

[1] results presented as n (%)

[2] Chi squared test comparing groups and the one tailed p value

Of the 42 different brands, altogether 22 (52.4%) did not have iodine levels within the recommendations and from the total number of samples only 64% of samples (15 crystal and 42 powdered salt) had the iodine levels within the specified range. It was important to note that of the 42 brands of salt only 2 had obtained the standard certificate from the Sri Lanka Standard Institute i.e., SLS certification.

Maternal iodine status

The median UI concentration of the sample in the 1st trimester was 175.2µg/L (IQR 106.3-263.4 µg/L) which was dropped to 126.0µg/L (IQR 74.8 - 196.4µg/L) at the end of 2nd trimester (Table 2). However, at the end of 3rd trimester, the median UI was further dropped to 106.0µg/L (IQR 67.4-160.6 µg/L) with a statistically significant reduction (p = <0.001).

Table 2: Urinary iodine concentration in the study sample

	1st trimester (n=425)	2nd trimester (n= 347)	3rd trimester (n=373)
Urinary iodine level (µg/L)[2]	175.2 (106.3; 263.4)	126.0 (74.8; 196.4)	106.0 (67.4; 160.6)
Wilcoxon Signed Ranks Test	Z=7.28; p<0.001	Z=4.11; p<0.001	p<0.001
	Z=10.39		

[1]Results presented as median (inter-quartile-range)
[2]Chi-square test at 2 df is 94.3; p = <0.001

The adequacy of UI levels in pregnancy was analyzed using the criteria given by WHO/UNICEF/ICCIDD, 2007 and tabulated in Table 3. It has shown that 177 (41.7%) mothers had insufficient UI concentration at the study entry. During the 2nd trimester, 204 (58.8%) were detected as having insufficient UI levels and at the 3rd trimester, 272 (72.9%) pregnant women had insufficient UI levels.

Table 3: Adequacy of urinary iodine excretion level during pregnancy[1]

Iodine Status[2]	1st Trimester	2nd Trimester	3rd Trimester
Excessive (≥500 µg/L)	4 (0.9)		2 (0.5)
Above requirements (250-499 µg/L)	120 (28.2)	37 (10.7)	24 (6.4)
Iodine Sufficient (150-249 µg/L)	124 (29.2)	106 (30.5)	75 (20.1)
Mild Iodine Deficiency (<150 µg/L)	159 (37.4)	168 (48.4)	232 (62.2)
Moderate Iodine Deficiency (<20-49 µg/L)	16 (3.8)	32 (9.2)	39 (10.5)
Severe Iodine Deficiency (<20 µg/L)	2 (0.5)	4 (1.2)	1 (0.3)
Total	**425**	**347**	**373**

[1] *Results presented as n (%)*
[2] *Iodine status (urinary iodine levels) was given by WHO/UNICEF/ICCIDD, 2007; "excessive" means in excess of the amount required to prevent and control iodine deficiency.*

According to palpation method 84.1% (n= 354) subjects did not have a palpable or visible goitre. Fifty five (13.1%) study subjects had a goitre that was palpable but not visible. Only 2.9% (n =12) had goitres which were not diagnosed previously.

The median thyroid volume was 5.16 mL (IQR 4.30; 6.10 mL) as measured by US scanning. The thyroid volume has a significant direct relationship ($p<0.001$) with the gland size as it increases steadily with the classification of goitre.

It was revealed that younger the mother (Age < 25 years) the thyroid volume is significantly ($p=0.04$) lower than the older mothers and mothers of their first pregnancy had significantly lower ($p<0.001$) thyroid volume than those were pregnant for the 2 time or more.

In this study the thyroid function tests were done to assess the iodine nutrition of subjects at the study entry (1st trimester) and at the end of the 3rd trimester. The Median serum TSH level of the subjects in their 1st trimester was 1.3mIU/mL (IQR 0.8 – 1.8 mIU/mL) and at the end of the 3rd trimester it was significantly increased ($p<0.001$) to 1.6 mIU/mL (IQR 1.2 – 2.1 mIU/mL). Median vales of fT_4 for 1st and 3rd trimesters were 18.0 pmol/L and 15.5 pmol/L ($p=0.002$) respectively.

There were no subjects with fT_4 values below the lower limit (<6.4pmol/L) of the reference (hypothyroid) range in both first and third trimesters.

A statistically significant association between UI levels and free T_4 levels in the third trimester (p=0.04) was observed. The pregnant women with thyroid size of grade 0 (n=354) had higher fT_4 (mean levels of 19.23 pmol/L) than the mothers of grade 2 (n= 12; mean level of 18.13 pmol/L). However, the difference observed was not statistically significant (p=0.74)

Maternal iron status

The mean Hb level at the study entry was 12.4 ± 0.92 g/dL and it dropped to 12.1 ± 1.1 g/dL by the third trimester (z test =4.47; p<0.001). The median SF level was 17.5 ng/mL (IQR 9.2; 30.0 µg/L) at the study entry and it improved to 30.3 ng/mL (17.4; 50.8 µg/L) at the third trimester (z test = 9.2; p<0.001).

It was noted that only 20 (4.8%) mothers were anaemic (Hb<11.0 g/dL) in the first trimester. However, the number of anaemic mothers increased to 49 (13.8%) at the end of the third trimester (p<0.001). In contrast, 178 (42.1%) pregnant women were iron deficient (SF <15.0 ng/mL) in the first trimester, but iron deficiency has been significantly (p<0.001) reduced as only 64 (17.5 %) pregnant women were having SF <15.0 ng/mL at third trimester (Table 4).

Table 4: Frequency distribution of Hb level and serum ferritin level

Characteristics	Trimester				Significance
	1st		3rd		
	n	%	n	%	
Haemoglobin Level (g/dL)					
Low (<11.0)	20	4.8	49	13.8	$\chi^2 = 19.13$
Normal (≥11.01)	396	95.2	305	86.2	df=1, p<0.001
Serum Ferritin Level (ng/mL)					
Low (<15.0)	178	42.1	64	17.5	$\chi^2 = 55.82$
Normal (≥15.01	245	57.9	302	82.5	df=1, p<0.001

In this study it was noted that 181 mothers (51.1%) obtained iron tablets ($FeSO_4$) from antenatal clinics and the remainder (n=171, 48.3%) consumed commercial preparations of iron tablet/capsules (Ferrous fumarate, gluconate, etc.) purchased from the private sector.

Of the 64 pregnant women who were iron deficient (SF<15.0 g/dL) at the third trimester, 31 (48.4%) obtained the iron supplements from Government antenatal clinics and the rest (n=33; 51.6 %) from the private sector. However there was no significant difference (p=0.92) in the number (%) of subjects with iron deficiency observed in the two categories.

The total content of iron consumed by each woman was calculated during pregnancy by the amount of elemental iron in each preparation and the number of days consumed. The pregnant women who received iron supplement from government clinics had a median intake of 10,080.0 mg (IQR 8,820.0; 10,500.0 mg) whereas those who purchased from the private sector had median intake of 8,400.0 mg (7,896.0; 8,750.0 mg). A statistically significant difference is seen in the two intakes (z test 1.94; p = 0.05).

The correlation coefficients on parameters at each investigation points are described in Table 5 (at the study entry) and Table 6 (at the third trimester). A significant negative correlation (r=-0.18; p<0.001) was seen between maternal serum TSH and fT_4 levels and significant positive correlation (r=0.12; p=0.01) between serum ferritin and Hb levels at the study entry. However, it was evident that in addition to these correlations there were significant negative correlations also (Table 6) between serum TSH and Hb (r=-0.14; p=0.01) and serum Ferritin with fT_4 (r= -0.19; p=0.01). No significant correlation between Tg and anaemia or iron status was seen in the first trimester.

Table 5: Correlation of the thyroid function test, anaemia and iron status in first trimester

	TSH	fT4	Hb	Ferritin	Tg
TSH	1	-0.18 (<0.001)	0.01 (0.79)	0.01(0.93)	0.05 (0.54)
fT4	-0.18 (<0.001)	1	-0.02 (0.71)	0.07 (0.16)	-0.01 (0.92)
Hb	0.01 (0.79)	-0.02 (0.71)	1	0.12 (0.01)	-0.06 (0.47)
Ferritin	0.01 (0.93)	0.07 (0.16)	0.12 (0.01)	1	-0.06 (0.52)
Tg	0.05 (0.54)	-0.01 (0.92)	-0.06 (0.47)	-0.06 (0.52)	1

Table 6: Correlation of the thyroid function test, anaemia and iron status in third trimester

	TSH	fT4	Hb	Ferritin
TSH	1	-0.11 (0.03)	-0.14 (0.01)	0.01 (0.89)
fT4	-0.11 (0.03)	1	0.05 (0.36)	-0.19 (0.01)
Hb	-0.14 (0.01)	0.05 (0.36)	1	0.12 (0.02)
Ferritin	0.01 (0.89)	-0.19 (0.01)	0.12 (0.02)	1

Effect of maternal iodine and iron status on neonatal thyroid function

The median (IQR) neonatal TSH (nTSH) level was 3.55 (2.50; 6.50) mIU/L whereas the median (IQR) UIC level was 105.20 (81.25; 142.00) µg/L. There were 4 infants with nTSH above the cut-off value of 20.00 mIU/L with the highest value of 39.00 mIU/L. However, all had normal serum TSH and fT_4 levels. Therefore, none of these babies were confirmed as having CH. There were 216 (62.3%) babies with nTSH<5.0 mIU/L and 37.7% of neonates had nTSH>5.0 mIU/L. Further, 285 (89.1%) had normal urinary iodine levels of ≥100 µg/L. Only 35 babies (10.9%) had insufficient (<100 µg/L) urine iodine levels.

A new born who has a birth weight of less than 2500g was considered as low birth weight infant (16 WHO, 2011a). The mean ±SD birth weight of the study sample was 3.01±0.49 Kg. The comparison of neonatal data with sex of the neonateis presented in Table 7. It was revealedthat the mean ±SD birth weight of males (3.10±0.46 kg) was significantly higher when compared to that of female counterparts (2.89±0.50kg; t-test 4.09; p<0.001). The median urinary iodine level in this sample was 105.5 (IQR 81.20; 142.0). The mean ±SD urinary iodine level among males (208.84±89.20µg/L) was significantly higher when compared with females babies (188.74±80.80µg/L; t-test 2.08; p=0.04).

The correlations between the measured parameters of the neonates with the maternal thyroid (serum TSH, fT_4, urinary iodine level and thyroid volume) and iron status (Hb and serum ferritin) were analysed and the results are tabulated in Table 8.The birth weight of infants showed a significant positive correlation (r=0.13; p=0.01) with mother's thyroid volume. Neonatal blood spot TSH level showed significant negative correlations with mother's 3rd trimester fT_4 level (r=-0.10; p=0.04) and Hb (r=-0.10; p=0.03) whereas the negative correlation with serum TSH (r=-0.01) did not reach to a significant level (p=0.80). Further, neonatal urinary iodine level had significant positive correlations with mother's 3rd trimester urinary iodine (r=0.23; p<0.001) and fT_4 (r=0.14; p=0.01) levels.

Table 7: General characteristics of the baby's in the study sample

Parameter	Unit	males	females	t-test	p-value
n		191	171		
Birth weight	kg	3.10±0.46	2.89±0.50	4.09	<0.001
Urinary iodine	µg/L	208.84±89.20	188.74±80.80	2.08	0.04
nTSH	mIU/L	5.20±3.60	5.39±4.60	-0.44	0.66

Table 8: Correlation between neonatal and maternal thyroid and iron parameters

		Baby's			Mother at 3rd trimester					
		BW	nTSH	nUI	UI	TSH	TV	Hb	SF	fT4
Baby's	BW		0.04	0.04	0.06	-0.01	0.13	0.02	-0.03	-0.02
			(0.45)	(0.47)	(0.27)	(0.94)	(0.01)	(0.68)	(0.57)	(0.72)
	nTSH	0.04		0.03	0.03	-0.01	-0.02	-0.10	-0.06	-0.10
		(0.45)		(0.60)	(0.55)	(0.80)	(0.75)	(0.03)	(0.30)	(0.04)
	nUI	0.04	0.03		0.23	0.02	-0.01	-0.03	-0.01	0.14
		(0.47)	(0.60)		(<0.001)	(0.79)	(0.87)	(0.62)	(0.92)	(0.01)
Mother at 3rd trimester	UI	0.06	0.03	0.23		-0.01	0.10	-0.01	0.01	0.01
		(0.27)	(0.55)	(<0.001)		(0.81)	(0.03)	(0.88)	(0.83)	(0.98)
	TSH	-0.01	-0.01	0.02	-0.01		-0.05	-0.01	0.01	-0.08
		(0.94)	(0.80)	(0.79)	(0.81)		(0.36)	(0.95)	(0.92)	(0.13)
	TV	0.13	-0.02	-0.01	0.10	-0.05		0.02	0.11	0.06
		(0.01)	(0.75)	(0.87)	(0.03)	(0.36)		(0.78)	(0.04)	(0.23)
	Hb	0.02	-0.10	-0.03	-0.01	-0.01	0.02		0.32	-0.01
		(0.68)	(0.03)	(0.62)	(0.88)	(0.95)	(0.78)		(<0.001)	(0.84)
	SF	-0.03	-0.06	-0.01	0.01	0.01	0.11	0.32		-0.14
		(0.57)	(0.30)	(0.92)	(0.83)	(0.92)	(0.04)	(<0.001)		(0.01)
	fT_4	-0.02	-0.10	0.14	0.01	-0.08	0.06	-0.01	-0.14	
		(0.72)	(0.04)	(0.01)	(0.98)	(0.13)	(0.23)	(0.84)	(0.01)	

Discussion

Iodized salt is the main source of iodine in Sri Lankan population and it has been the main strategy to control the IDD. It was reported that even in iodine sufficient or mildly iodine deficient (MID) areas, iodine deficiency during pregnancy frequently appears and thyroid gland cannot meet the demand for increasing the production of thyroid hormones. Its effect may be damaging the neurodevelopment of the foetus. Therefore, it is important to prevent even mild iodine deficiency during pregnancy to have a better pregnancy outcome. Investigations on iodine nutrition in pregnancy in Sri Lanka were very few. No national level studies have been conducted to assess the iodine nutrition in pregnant women in Sri Lanka up to 2010. The study carried out by MRI in 2010 revealed that the overall median urine iodine concentration among pregnant women was 113.7µg/L indicating an iodine deficiency in Sri Lankan pregnant women (9) and it was not a follow up study. The present study analyzed the maternal iodine status prospectively in detail using thyroid size, thyroid profile and UI level.

In the present study it was clearly evident that the median UI concentration reduced significantly during the course of pregnancy ($p< 0.001$). This progressive decrease in urine iodine concentration can be attributed to the increased demand of iodine as a result of advancement of the pregnancy [17]. Indeed, the apparent better iodine status in early pregnancy may be due to the increase of glomerular filtration during first trimester causing an increased UIC and, therefore an increased loss of iodine from the body. The reduction in urinary iodine level with the advancement of gestational age in this study is quite compatible with the results of studies done in Bangladesh [18], Congo [19] and Nigeria [20]. In contrast results of national health and nutrition examination survey (NHANES) in USA showed that UI levels were increased in the second and third trimesters when compared to the first trimester [21]. This increase in iodine status was attributed to the usage of supplements containing iodine during pregnancy.

Present study showed that only 64% of salt products contained iodine within the recommended range and a previous investigation too [9] showed that household usage of adequately iodized salt was 69.4%. These factors may also be contributing to the poor iodine nutrition status evident

during pregnancy in the study population and it suggests that the consumption of iodized salt has not met the increased demand for iodine during pregnancy in this study population.

The increase in serum TSH level and decrease in fT_4 level with the advancement of pregnancy in our study was compatible with the findings of the study done in Bangladesh [18]. The UI by the TSH levels in both first and third trimesters, showed that vast majority of the subjects had TSH levels within normal range irrespective of the UIC in both first and third trimesters. Even though UIC decreased towards the end of the pregnancy serum TSH levels were maintained within the reference range.

The median fT_4 level was significantly reduced from18.0pmol/L (IQR 14.2-21.9) in the first trimester to15.5 pmol/L (IQR 11.6 - 21.9) towards the end of the pregnancy (p=0.002). Even though $fT_4$was not a good indicator of iodine nutrition during pregnancy, normal levels indicated that the thyroid function was maintained properly in the study sample. There was an association between thyroid size and mean fT_4 levels: women with thyroid size of grade 0 (n=351) had higher fT_4 (mean levels of 19.23pmol/L) than the women of grade 2 (n= 12; mean level of 18.13pmol/L). However, this observed difference was not statistically significant (p=0.74). Other reasons for goitre (immune markers) were not evaluated in this study.

Even though iodine nutrition among the study population was poor based on the UI status, the results of the thyroid function tests appears otherwise. Urine iodine levels in the first and third trimesters did not show any significant association with thyroid function (TSH and fT_4) tests. One of the most probable reasons may be not using the reference ranges for the thyroid function test specific to pregnancy, and there must be a concrete agreement regarding the method and the reference ranges to be used in the assessment of thyroid functions during pregnancy in our population.

Studies in Sri Lanka have indicated different prevalence rates for anaemia during pregnancy. It was common to see from findings of many of the studies that prevalence rates of anaemia during pregnancy was relatively low when compared to the national figures [22,23]. The present study also showed low prevalence rate (4.8%) in contrast to the figure of 16.7% given in the most recent national survey [24].

The usual trend that was observed in relation to the iron stores in pregnant women was the depletion of iron stores towards the end of the pregnancy [25, 26, 27]. Senanayake *et al.*, in 2010 (28) showed a similar result in a study done in Sri Lanka. In contrast to these studies a significant improvement in ferritin level seen in this study sample illustrates that the iron stores of the overall sample has been improved towards the end of the pregnancy even though the prevalence of anaemia was increased. It may be due to the successful iron supplementation programme carried out in Sri Lanka at present.

It was observed that almost equal percentage of pregnant women received iron supplements either from government or private sectors. Further, there was no difference in the proportion of anaemia and iron deficiency observed in the two groups. The commercial preparations are much more expensive than the $FeSO_4$ given in the government sector. The results showed that almost half of the pregnant women in this sample preferred commercial preparations obtained at a very high cost despite the fact that iron supplements are freely available at the antenatal clinics conducted by the Health Department. The most probable reason for that may be the common perception among some medical personnel and the pregnant women that commercial iron preparations are better in improving iron status and it has been proven wrong by this study.

It has been suggested that those who are iodine deficient may also be iron deficient and if IDA is a nutritional factor that influences the pathogenesis of IDD, it may have a greater impact on IDD than goitrogens because of its high prevalence in vulnerable groups [29]. The requirement for thyroid hormone during pregnancy sharply increases [30] and it is obvious that concurrent iron deficiency may further impair the maternal thyroid function in iodine deficient pregnant women. Assessing urine iodine level as a measure of iodine nutrition in new born is a very difficult task and it has not been done in Sri Lanka. The main challenge in measuring urine iodine is the difficulty in collecting urine samples from the neonate [31].

The study done at Turkey showed that 10.3% of the newborns and 56.8% of mothers were iodine deficient [32]. A recent study in Iran revealed that prevalence of neonatal iodine deficiency was 14.2% and the urine iodine level was < 100 µg/L in 33.9% of mothers (33). A common finding in these studies including the present study is the lower prevalence of iodine deficiency among neonates despite the higher prevalence of maternal iodine deficiency. Urine iodine level among neonates in the present study had a significant positive correlation with mother's 3rd trimester urinary iodine ($r=0.23$; $p<0.001$) similar to the Iranian study ($r=0.46$, $P<0.001$) [33].

The results of the present study showed that 37.7% (n=131) of neonates had TSH level >5.0 mIU/L. It has been shown in Thailand that 8.9% of neonates had TSH >5.0mIU/L (34). A neonatal TSH frequency of >5.0mIU/L has been reported in less than 3% of neonates in several mildly iodine deficient regions suggesting that neonatal TSH may not be sensitive enough to evaluate iodine status when there was mild iodine deficiency. A study from Australia using a sensitive TSH assay found that only 2.2% of neonates had a TSH value >5.0% despite a median UIC of 85.0 µg/L among pregnant women [35].

When assessing the iodine nutritional status, it has been proposed that neonatal thyroid-stimulating hormone (TSH) concentration is a good indicator of iodine deficiency in the population. The WHO has proposed to use the results of screening programmes for congenital hypothyroidism in neonates as an additional index for the evaluation of iodine status of the population. A frequency of neonatal TSH concentrations > 5.0mIU/L below 3% was proposed as indicating iodine sufficiency (36). In mild iodine deficiency the frequency may be 3.0–19.9%. The frequencies of 20.0–39.9% and above 40% may be found in moderate and severe iodine deficiency respectively The iodine deficiency in the present study was moderate according to WHO criteria [36] that the median (IQR) neonatal TSH level was 3.55 (2.50; 6.50) mIU/L. These findings are similar to the data from elsewhere (37). A study done in Thailand showed that the mean neonatal TSH was 2.40 (SD 1.56) mIU/mL and according to them, although the median neonatal TSH concentrations were within normal ranges, the proportion with TSH >5 mIU/L ranged from 6.0-14.0%, an indication of iodine insufficiency in both mothers and fetuses during pregnancy [34].

In the present study we studied the correlation of neonatal TSH with maternal TSH and fT_4 level levels. The blood spot TSH level showed significant negative correlations with mother's 3rd trimester fT_4 level (r=-0.10; p=0.04) whereas the negative correlation with serum TSH (r=-0.01; p=0.80) did not reach a significant level. A recent study also showed that neonatal TSH is not associated with maternal thyroid function in either of the two trimesters studied [38].

There were evidences to suggest that iron deficiency affect the thyroid functions [39, 40] and therefore the effect of maternal iron status on neonatal thyroid function was assessed in the present study. According to the results, there was a negative correlation between neonatal TSH and third trimester serum ferritin level (r=-0.16; p=0.30) though not significant, but the blood spot nTSH level showed a significant negative correlation with mother's 3rd trimester Hb level (r=-0.10; p=0.03). The clinical relevance of this significant negative correlation is not clear but it indicated that maternal iron status does not influence the neonatal thyroid function.

Conclusions

Although iodization of salt is compulsory by law in Sri Lanka it appears to be not well monitored as significant number of salt products do not contain recommended levels of iodine. A proper monitoring system at the production level to assess iodine concentration appears important.

About three fourth of pregnant women were iodine deficient in the third trimester and out of that, the majority had mild iodine deficiency. This finding quite agrees with the recent findings of iodine nutrition of pregnant women in Sri Lanka. The effectiveness of iodized salt in maintaining iodine nutrition during pregnancy is questionable and it suggests that iodized salt consumption is not meeting the increased demand for iodine.

The overall maternal iron status was satisfactory. Effect of maternal iron status on maternal thyroid function was not significant. Higher prevalence of iron deficiency in early pregnancy should be addressed.

Prevalence of iodine deficiency among newborn in this sample is relatively low (10.9%) in contrast to maternal iodine deficiency. Neonatal UI level indicated significant positive correlation with third trimester maternal UI level and maternal fT_4 but the correlation between neonatal TSH and maternal UI level was not significant in this study. Higher prevalence of neonates with TSH >5 mIU/L (37.7%) indicates moderate iodine deficiency during pregnancy and in neonates in this study sample but interpretation should be done carefully. Maternal iron status does not influence the neonatal thyroid function in this study sample.

Acknowledgement

I wish to thank MOH Bope- Poddala health division and staff of the Teaching Hospital, Mahamodara, Galle, for the support given for the subject recruitment and sample collection. Mrs. R. Karunathilaka, Mr. Priyanka Bandara Attanayake and Mr. Sanath Dharmapriya, Faculty of Medicine ,University of Ruhuna for technical assistance. A special thank is extended to Dr. Manjula Hettiarchchi, Professor Chandrani Liyanage and Professor K.A.P.W. Jayathilaka for supervising the study. I wish to acknowledge TURIS project University of Ruhuna for the financial assistance provided for the project.

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Ultra-Bright Metal-Fluorophore Aggregates by Metal-Enhanced Fluorescence of Dye-Doped Silica Nano Particles

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ABSTRACT

Enhancement of molecular fluorescence is of great interest due to the widespread popularity of fluorescence-based detection techniques available today. Although fluorescence-based detection is considered to be more sensitive than other optical approaches, there is still an intense need for more photo stable, high quantum yield fluorophores. In this regard, metal-enhanced fluorescence (MEF) has opened novel pathways for the development of brighter, fluorescent markers with enhanced stability. Surface plasmon resonance of metal nanoparticles can modify the radiative properties of nearby fluorophores in ways not seen in classical fluorescence. Trapping fluorophores between coupled plasmons can further enhance the near-field interactions leading to even larger enhancements. The aim of this research was to a develop a solution based approach to study MEF by aggregation of fluorophore tethered gold nanoparticles, which would ultimately lead to the development of ultra-bright fluorescent probes. Here, we report a simple method for aggregating multiple gold nanoparticles (GNPs) on Rhodamine B (RhB)-doped silica nanoparticles (SiNPs) utilizing dithiocarbamate (DTC) chemistry to produce MEF in solution. Dye was covalently incorporated into the growing silica framework via co-condensation of a 3-aminopropyltriethoxysilane (APTES) coupled RhB precursor using the Stöber method. Electron microscopy imaging revealed that these mainly non-spherical particles were relatively large (80 nm on average) and not well defined. Spherical core-shell particles were prepared by physisorbing a layer of RhB around a small spherical silica particle (13 nm) before condensing an outer layer of silica onto the surface. The core-shell method produced nanospheres (~30 nm) that were well defined and monodispersed. Both dye-doped SiNPs were functionalized with pendant amines that readily reacted with carbon disulfide (CS_2) under basic conditions to produce DTC ligands that have exhibited a high affinity for gold surfaces. GNPs were produced via citrate reduction method and the resulting 13 nm gold nanospheres were then recoated with an ether-terminated alkanethiol to provide stability in ethanol. Fluorescent enhancement was observed when excess GNPs were added to DTC coated dye-doped SiNPs to form nanoparticle aggregates. Optimization of this system gave a fluorescence brightness enhancement of over 200 fold. Samples that gave fluorescence enhancement were characterized through Transmission Emission Micrograph (TEM) to reveal a pattern of multiple aggregation of GNPs on the dye-doped SiNPs.

Introduction

Fluorescence spectroscopy is a widely used technique in medical diagnostics and in biological research. During the past few decades applications of fluorescence in biology have undergone extensive development due to the increasing availability of fluorescent proteins, dyes, and probes that enable the non-invasive study of gene expression, protein function, protein-protein interactions, and a large number of cellular processes [1-5]. Few of such applications are cellular imaging, DNA sequencing, fluorescence tomography and molecular beacons. Although fluorescence-based detection is considered to be more sensitive than other optical approaches there is still a need for the development of more photo stable, high quantum yield fluorophores, which would greatly enhance the effectiveness of techniques in single molecule detection [6, 7], cellular tracking and imaging [8], as well as the miniaturization of optical sensors [9, 10].

Most applications in classical fluorescence spectroscopy take advantage of radiation emitted to the far field which means radiation detected at least several wavelengths away from the fluorophore. In recent years there has been a growing interest to study the near field interaction between fluorophores and metallic particles in the nano meter regime. Noble metal particles in the nanometer regime exhibit unusual characteristics which are not displayed by the bulk metals or by individual atoms. The most attractive of them is their color which results due to a strong absorption band in the visible region. For example, silver nanoparticles (AgNPs) absorb at 450 nm whereas gold nanoparticles (AuNPs) absorb at 520 nm. The characteristic color of metal nanoparticles results due to absorption of electromagnetic radiation which causes collective oscillations of surface electrons on nanoparticle surface which is known as surface plasmon resonance (SPR). When an excited fluorophore is placed within wavelengths distance from a metal particle, near field interactions that occur between the electron clouds (plasmon) of the metal particle and the excited fluorophore can dramatically change the spectral properties of the fluorophore. This fairly new field in plasmonics is called Metal-Enhanced fluorescence (MEF).

MEF is a powerful technique which can simultaneously increases photo stability and brightness of fluorescent molecules [9, 11-14]. This effect is highly sensitive to the distance between the nanoparticle and the fluorophore (~5-30 nm) [15]. If the fluorophore is less than 5 nm away from the metal, plasmonic effects could quench the fluorophore. Therefore, fluorescence enhancement is highly dependent on the distance. Furthermore, MEF can be influenced by the size, shape, and aggregation of the metal particles as well as the spectral overlap of fluorophore and the plasmon. In particular, it has been shown both theoretically [19-23] and experimentally, that the overlap of multiple plasmons of nearby metal colloids would strongly intensify the electromagnetic field in the overlapped region and a single fluorophore located in this region has shown greater brightness

enhancements [24-27]. Enhancements greater than 50 fold have been reported for fluorophores next to multiple silver nanoparticles with large scattering cross sections [19]. However, these MEF techniques required stationary immobilization or bio-molecule tethering that are most practical for surface assays [26].

In this work we report a simple solution-based approach for aggregating multiple gold nanoparticles (GNPs) around Rhodamine B (RhB)-doped silica nanoparticles (SiNPs) using dithiocarbamate (DTC) attachment. Organic dye-containing (doped) fluorescent SiNPs exhibit many advantages for biological applications. They are biocompatible, non-toxic, highly hydrophilic, optically transparent, size-tunable and can be easily modified with various biomolecules. Gold nano-particles are also attracted to the scientific community due their biocompatibility and surface funtionality. In this work we aimed to achieve large enhancement factors of the fluorophore; which is Rhodamine B (RhB)-doped silica nanoparticles by placing them at an optimal distance from the gold surface as well as aggregating multiple gold nanoparticles around a small number of dye molecules. We have used dye-doped SiNPs as solution-suspended fluorescent nano-platforms. The silica framework allowed a fixed distance to be created between the dyes and the metallic surface and provided a platform for multiple GNPs to be attached in solution. The resulting extremely bright metal-fluorophore aggregates support a greater potential of creating MEF using solution-based methods and can be used as ultra-bright, photo-stable fluorescent markers in biological applications.

Materials and Methods

For this study dye-doped SiNPs were produced by two methods. In method 1, modified Stöber method was used to prepare dye-doped SiNPs. SiNPs were produced by co-condensing the siloxane-tethered RhB units (1.5 mL in DMF) with tertaethoxyorthosilicate (TEOS) (0.335 mL) in a 0.06:1.5 molar ratio in the presence of pure ethanol (8.75 mL) and ammonium hydroxide (NH_4OH), (0.64 mL) for 24 h [30]. Two batches of dye doped SiNPs with different dye concentrations were produced by Stöber method. Particles doped with high concentration of dye were produced by mixing siloxane-tethered RhB units with NH_4OH for 12 h prior addition of TEOS. Particles doped with low concentration of dye were produced by mixing the above two components for 5 minutes prior addition of TEOS. This co-condensation method produced amorphous particles that presumably contained RhB dye dispersed throughout the SiNP [31]. Based on the mass balance of these condensation reactions, we could estimate the concentration of the silica particles in stock aqueous suspensions. We examined these SiNPs by transmission electron microscopy (TEM) to determine that they were amorphous particles with a cross section of 100 nm. TEM imaging was performed on a Zeiss 10A Conventional microscope operating at 80

kV from a tungsten filament source at the University of Oklahoma Samuel Roberts Noble Electron Microscopy Laboratory. Samples were prepared by drop coating 4 µL of sample onto a 300 mesh formvar coated copper TEM grid (Ted Pella Corp.) and allowing to dry under ambient air.

In method 2, a core-shell design was used to hold the dye at a set distance from the metal surface by sandwiching layer of dye between two concentric spheres of silica [32]. The two types of dye-doped SiNPs are illustrated in Fig. 1. Layered SiNP were prepared to control both the size of the spheres and the location of flourophore within the silica matrix. TEM imaging confirmed the production of 16 nm spherical SiNPs. Cationic RhB dye was then physisorbed in an aqueous suspension onto the surface of these well-defined core particles [32]. Finally, an additional layer of silica was grown by a lysine catalyzed condensation of TEOS in an aqueous organic biphasic system to provide an outer shell for small diameter layered SiNPs. The dye-doped particles were purified by membrane dialysis (12-14 kD MWCO) against milipore water. Dye loading was found to be 95% based on the absorbance of the dialysis water to determine the amount of fluorophore that was not incorporated. TEM imaging confirmed the formation of monodisperse 30 nm SiNPs. The optical absorption and emission spectra of both stock SiNP mixtures produced by the two different methods were measured. Absorbance measurements were taken on a Shimadzu Scientific UV-2101 PC UV-Vis scanning spectrophotometer. Fluorescence measurements were taken on a Shimadzu Scientific RF-5301 PC spectrofluorophotometer equipped with a xenon lamp of 150W as an excitation source.

Gold nanoparticles were produced via the well-established reduction of chloroauric acid in aqueous sodium citrate [33, 34]. The resulting citrate-stabilized 13 nm gold nanospheres were then recoated with (10-(2-(2-methoxyethoxy)ethoxy)decane-1-thiol $CH_3O(CH_2CH_2O)2C_{10}H_{20}SH$), an ether-terminated alkanethiol to provide stability in water/ethanol mixture [36]. Recoating of GNPs with the ether-terminated alkanethiol was optically characterized by the slight red shifting of the 520 nm UV/Vis spectra of the GNP plasmon. The concentration of the stock GNP solution was then estimated using the extinction coefficient [37, 38].

Establishing a robust method for attaching gold and silica particles together is vital to optimizing MEF as the enhancement factor rises with increased aggregation. Wie et al have demonstrated that dithiocarbamate (DTC) effectively stabilizes ligands on gold surfaces [39]. Recently, DTC chemistry has been used to immobilize gold nanoparticles on amine coated bulk silica substrates [40]. This method was modified to covalently bind SiNPs to GNPs in solution (Fig. 2). For DTC activation, the surface of the SiNPs were amino funtionalized and then mixed with CS_2 under basic conditions. Particles produced by the modified Stöber method were initially used for DCT activation. Initially the surface of RhB doped SiNPs was amino-propyl functionalized, then particles were DTC activated by adding varying amounts of amino-propyl functionalized SiNPs (250, 25, 12.5, 10 µL) to CS_2 solutions (1 mM, 3 mL) prepared in 95 %

ethanol. The pH of the samples was raised to 9.8 by drop-wise addition of concentrated KOH and the samples were stirred for 30 min to complete DTC activation in capped glass vials. For nano particle aggregation, ether-terminated alkanethiol capped GNPs (1.0 mL, 1.2 nM) were added to the DTC-activated SiNP mixtures and stirring was continued for another 24 h. Above SiNP and GNP amounts were chosen to approximately provide desired SiNP to GNP ratios to be 1:1, 1:10 and 1:20, absorbance and emission of the samples were measured over time. Enhancement was measured by comparing the emission spectra of RhB-doped SiNP control samples that lacked GNPs. The amount of aggregation was compared relative to absorption spectra of SiNP and GNP mixtures lacking CS_2 so that DTC formation was prohibited. Once the amount of SiNPs that exhibited enhancement was screened, enhancement was further optimized by varying the GNP concentration. Experiment was repeated with SiNPs having high and low concentrations of dye loadings produced by the Stöber method.

Layered dye-doped SiNPs produced by Method 2 were similarly treated. However, the concentration of the stock solution of the layered particles was difficult to obtain as these particles did not efficiently centrifuge out of solution. Therefore, an aliquot that provided the same fluorescence emission as in method 1 was used. The same procedure described for method 1 was used for APTES coating and DTC activation of layered SiNPs. Then gold nano particles were added and the samples were mixed continuously to form metal-fluorophore aggregates. The absorbance and emission were measured over time. Enhanced samples of metal-fluorophore aggregates formed by SiNPs from method 1 and method 2 were imaged through TEM to study the aggregation pattern.

Results and Discussion

Dye-doped SiNPs produced by two methods were interacted with GNPs to study MEF. Calculation of the concentration of GNPs was easily performed using absorbance measurements. Concentration of the SiNPs produced by the Stöber method was estimated based on the mass balance of these condensation reactions of the aqueous suspensions. Unfortunately, the same could not be done for the layered dye-doped SiNPs. The relative amount of dye molecules absorbed into a sphere could be estimated but extending this approximation to estimating the concentration of SiNP proved unhelpful. Both Stöber and layered particles were initially functionalized with the amino-propyl ligands that were subsequently converted to DTC by reaction of CS_2 under basic conditions. Ether-terminated alkanethiol capped GNPs were then added to DTC activated dye-doped SiNPs to produce metal-fluorophore aggregates. Both SiNP species showed GNP aggregation, which lead to large enhancements in fluorescent brightness [41, 42].

To achieve fluorescent enhancement it was necessary to optimize the concentrations of the dye-doped SiNPs and GNPs as well as to establish a robust aggregation motif. Using dye-doped

Figure 1: Two motifs for RhB incorporation into silica nanoparticles. (a) Amorphous particles with covalent dye attachment throughout the entire SiNP via 3-aminopropyltriethoxysilane (APTES) bound RhB. (b) Spherical particles with RhB dye shell formed by physisorption in layered silica preparation.

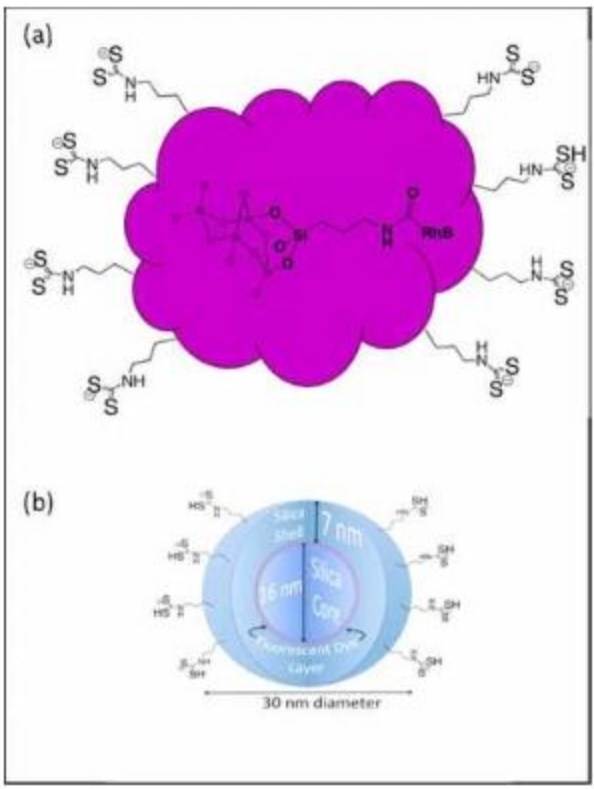


Figure 2: Conversion of amino-propyl coated silica nanoparticles to dithiocarbamate (DTC) thus, promoting aggregation with gold nanoparticles.

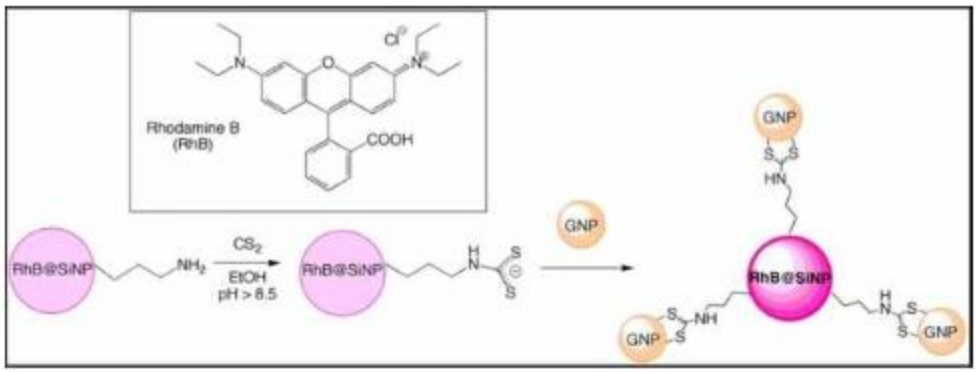


Figure 3: Emission spectra of DTC activated RhB-doped silica nanoparticles mixed with gold nanoparticles over the course of seven days. Inset shows the emission spectra of control samples.

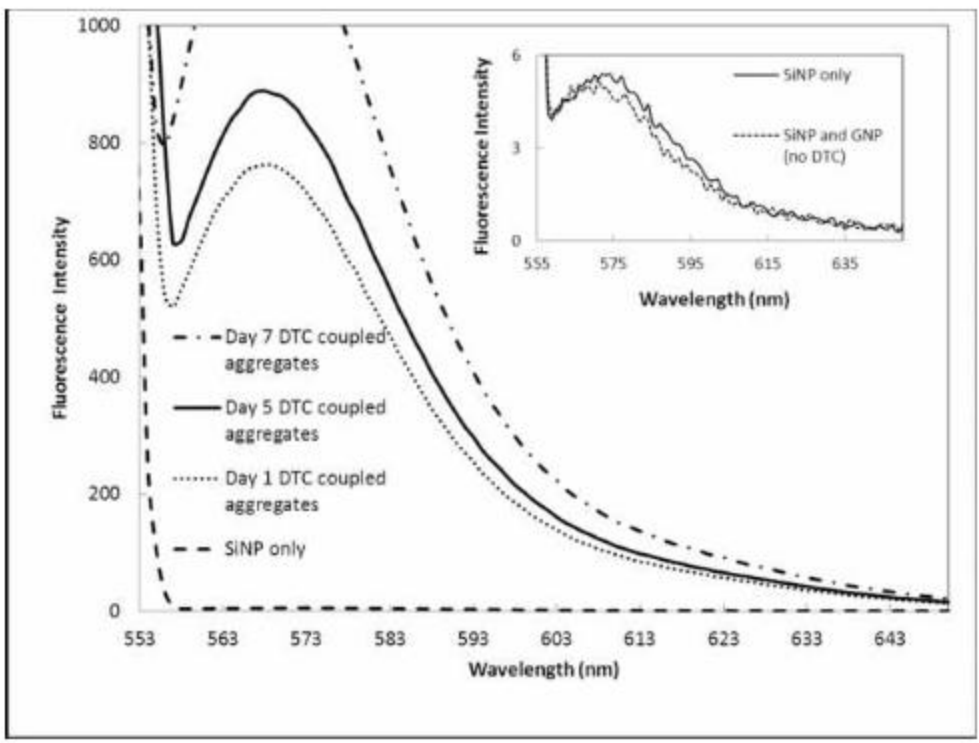


Figure 4: Absorption spectra of DTC activated RhB-doped silica nanoparticles mixed with gold nanoparticles over the course of seven days.

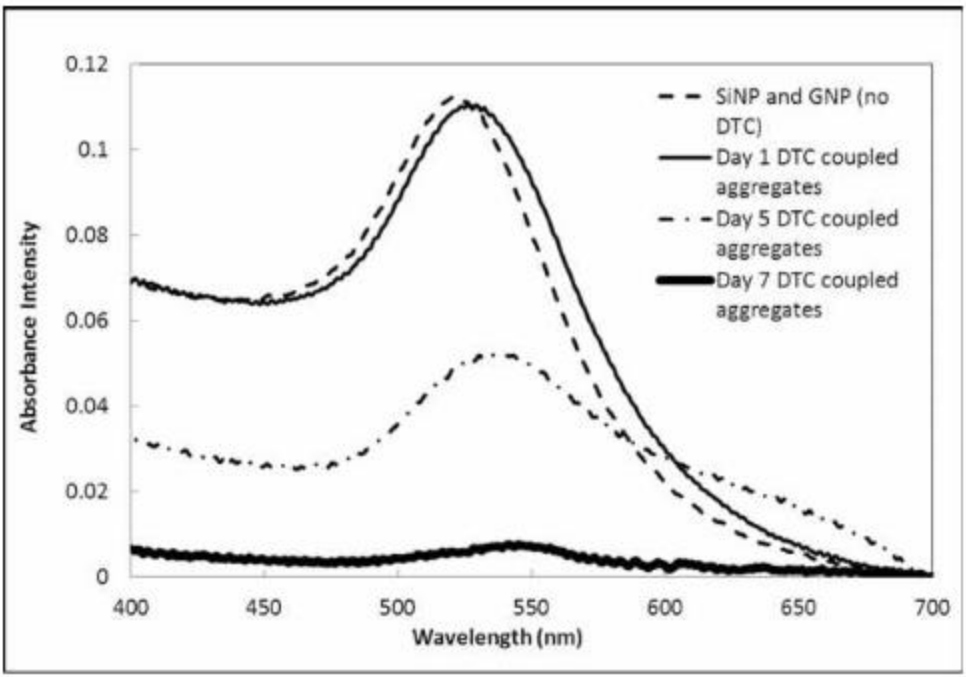
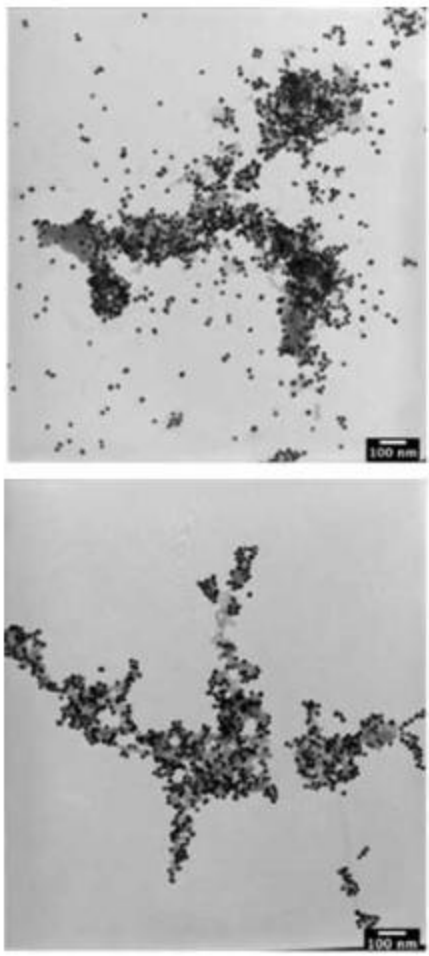


Fig. 5 TEM micrographs of gold nanoparticle and RhB-doped, DTC activated silica nanoparticle mixtures; (a) 2 days (b) 14 days after mixing.



SiNPs produced by method 1, different ratios of ether-terminated alkanethiol capped GNPs were mixed with DTC activated SiNPs in water/ethanol mixture to form metal-fluorophore aggregates. The fluorescence and absorbance of the mixtures were measured over time. Optical enhancement of experimental samples was relative to the fluorescence emission of SiNPs in the absence of GNPs. The aggregation of the nanoparticles mixtures due to DTC activation was determined by monitoring the red shifting of the absorbance spectra as an indication of interacting GNPs [43]. The enhancement process proved to be very sensitive to the ratio of SiNPs to GNPs. While no

enhancement was observed when GNPs and SiNPs produced by method 1 were mixed at a ratio of 1:1, an increase in brightness of fluoresence at higher GNP concentrations was observed. A significant increase in enhancement was also observed when the concentration of RhB dye was lowered within the SiNPs. This result is consistent with a reduction of fluorophore auto quenching that is observed for highly concentrated samples of RhB [44]. The fluorescence enhancement of these particles varied over time, increasing from about 100 fold after the first day to 200 fold after 7 days (Fig. 3). The absorbance spectra also red shifted (Fig. 4) as the fluorescence continued to increase over time. The increased red shift is consistent with increased GNP aggregation. A variation in the aggregation of GNPs around the amorphous dye-doped SiNPs was studied by TEM imaging.

TEM studies of these samples show an increasing degree of SiNP/GNP aggregation over the course of several days (Fig. 5). These images are in excellent agreement with the observed extinction spectra that show a gradual decrease in gold plasmon absorption (Fig. 4) as the amount of colloidal gold decreases. This supports the theory that fluorescent enhancement is due to GNP aggregation, which leads to increased plasmonic scattering [45, 46].

Treatment of the layered dye-doped SiNPs produced by method 2 was similarly studied. GNP aggregation was once again demonstrated concurrently with the increased emission (Fig. 6). Formation of aggregates was confirmed by red shifting of the absorbance spectra, the emission spectra showed about 50-fold enhancement on day 1 and about 100-fold enhancement after 8 days. TEM images of these samples further confirmed formation of metal-fluorophore aggregates.

One limitation of this solution-based approach is the difficulty in controlling the aggregation in solution phase. It was difficult to reproduce the same amount of metal-fluorophore aggregation in each trial due to the inherent randomness associated with solution-based complexation. Thus, it was difficult to reproduce the fluorescence enhancement to the same level in each trial. However, we were able to reproduce enhancement in different amounts in several trials.

Conclusion

In this study we have described a simple solution based approach to achieve metal-enhanced fluorescence by aggregation of a large concentration of gold nanoparticles around a smaller concentration of RhB doped silica nanoparticles. RhB doped SiNPs were produced by two methods; first method formed larger amorphous particles having dye throughout and the second method yielded smaller, more spherical particles having RhB dye sandwiched between two layers of silica. DTC activated dye-doped SiNPs were were mixed with gold nanoparticles to form metal fluorophore aggregates. Absorbance spectra confirmed that both kinds of SiNPs showed metal-fluorophore aggregation and the aggregation increased over time. We achieved 100-fold

fluorescence enhancement on the first day, which increased up to 200 fold over period of seven days. TEM data was used to further confirm metal-fluorophore aggregation. This novel technique allows the study of MEF by a simple solution based approach using SiNPs as fluorescent nano platforms. Employment of DTC chemistry for metal fluorophore aggregation simplifies the need for biomolecule tethering in study of MEF. Further studies are required to investigate the control of degree of aggregation in order to harness the maximum effect of metal enhanced fluorescence technique.

Acknowledgements

We thank the National Science Foundation (NSF) for funding this work (DMR – 0805233). We thank Anuradha Singh for providing S-10-[2-(2 methoxyethoxy)ethoxy]decyl ethanethioate ($CH_3O(CH_2CH_2O)C_{10}H_{20}SOCH_3$) for GNP surface functionalization.

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