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Abstract

Objective: Selenium is effective in improving quality of life and reducing the progression of active Graves' orbitopathy. The effect of correcting relative selenium deficiency on improving Graves' orbitopathy is unknown, as baseline selenium levels have not previously been measured. The study aims to determine if serum selenium levels are reduced in patients with Graves' orbitopathy (GO) compared with Graves' without orbitopathy (GD).

Design: A prospective, case-control study performed between 2009 and 2012 at endocrine and ophthalmology clinics in Australia.

Patients: A total of 198 patients with Graves' disease participated in the study: 101 with Graves' orbitopathy and 97 without Graves' orbitopathy.

Measurements: Serum selenium levels in both groups.

Results: Mean serum selenium levels were significantly lower in GO ($1.10 \pm 0.18 \mu\text{mol/L}$) than GD ($1.19 \pm 0.20 \mu\text{mol/L}$) ($P=0.001$). Mean selenium levels appeared to decrease in parallel with increasing severity of GO; selenium level was $1.19 \pm 0.20 \mu\text{mol/L}$ in GD, $1.10 \pm 0.19 \mu\text{mol/L}$ in moderate to severe GO and $1.09 \pm 0.17 \mu\text{mol/L}$ in sight-threatening GO ($p=0.003$). Serum selenium levels remained significantly lower in GO after adjusting for age, smoking status, thyroidectomy, radio-active iodine treatment and residential location.

Conclusion: Serum selenium levels are lower in patients with GO compared with GD in an Australian study population with marginal selenium status. Relative selenium deficiency may be an independent risk factor for orbitopathy in patients with Graves' disease.

Introduction

Selenium, an essential trace element, has a number of actions, including the production of active thyroid hormone and as an anti-oxidant and anti-inflammatory agent. Selenium concentrations are highest in the thyroid gland.¹ Reduced selenium levels have been reported in patients newly diagnosed with autoimmune thyroid disease,^{2, 3} Multiple prospective randomized control trials showed successful serial reduction of thyroperoxidase (TPO) autoantibodies with selenium supplementation in Hashimoto's thyroiditis after 3-12 months.⁴⁻⁷ whilst other studies did not show significant changes in TPO autoantibodies levels or thyroid function with selenium supplementation.^{3, 8}

A randomized trial by the European Group on Graves Orbitopathy showed selenium is effective in improving the quality of life and reducing the progression of mild active Graves' orbitopathy.¹⁰ The mechanism of improving Graves' orbitopathy remains unclear, but one possibility is an effect mediated by reduction in oxidative stress, as the selenoproteins protect against damage caused by reactive oxygen species. Correcting selenium deficiency could also explain its effect on improving Graves' orbitopathy. This hypothesis is currently untested because baseline selenium levels have not previously been measured in Graves' orbitopathy (GO) compared with Graves' without orbitopathy (GD). We aimed to determine whether serum selenium concentrations are reduced in patients with GO compared with GD in a prospective, case-control study.

Patients and Methods

Patients diagnosed with Graves' disease were prospectively recruited from multiple endocrine and ophthalmology clinics in Victoria, Australia from 2009 to 2012. All participants were examined for the presence and severity of thyroid orbitopathy by an ophthalmologist using VISA classification.¹¹ Patients were interviewed to determine their

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ethnicity, age, address by postcodes, treatment for Graves' disease and thyroid orbitopathy, smoking status, family history and thyroid-specific ophthalmic symptoms. Thyroid function tests including thyroid stimulating hormone (TSH), free tri-iodothyronine (fT3), and free thyroxine (T4), and TSH receptor antibody levels were obtained from medical records.

This prospective, case-control study was approved by each site's human research ethics committees. Moderate and severe GO were included as cases. Cases either had sight-threatening complications (optic neuropathy, exposure keratopathy), thyroid orbitopathy requiring surgical rehabilitative surgery (orbital decompression, lid recession, blepharoplasty, strabismus surgery), or untreated thyroid orbitopathy with moderate to severe appearance on examination. Controls (GD) had Graves' disease for more than 2 years, without clinical signs or symptoms of thyroid orbitopathy. Participants' locations of residence were defined into metropolitan and non-metropolitan categories according to Australian Standard Geographical Classification 2011, according to statistical division code 05.

Blood samples were collected and centrifuged and serum was stored at -80°C . Serum selenium was measured using graphite furnace atomic absorption spectrophotometry and compared between cases and controls. The method uses an AAnalyst 800 Atomic Absorption Spectrophotometer (Perkin Elmer, Glen Waverley, Victoria, Australia) with a graphite furnace and a specific selenium electrode discharge lamp. Blood samples from 217 Victorian blood donors were used to establish a reference interval for adults ($0.8\text{-}1.4\mu\text{mol/L}$). There were no observed differences in selenium levels due to age or sex.

Statistical analysis

Statistical analysis was performed using Minitab v16. Serum selenium levels were normally distributed in cases and controls, hence a two-sample T test was used to compare the differences in selenium levels between GO and GD; $P < 0.05$ was considered significant.

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Mean serum selenium levels in GD, moderate to severe GO and sight threatening GO were analysed using one-way ANOVA. Thyroid function tests and TSH receptor antibody levels were not normally distributed, hence medians were compared using non-parametric Kruskal Wallis tests between cases and controls. We used the chi-square test to determine the statistical significance for differences noted in categorical variables. We used binary logistic regression with GO as the outcome for univariate analysis including selenium level, thyroid function tests, TSH receptor antibody, age, gender, smoking status, ethnicity, residential location, duration of Graves' disease, anti-thyroid medication use, radioactive iodine treatment and thyroidectomy. Only variables associated with GO in univariate analyses were entered into a multivariate model, variables with $p > 0.05$ were eliminated in stepwise analyses.

Results

198 patients were included in the study. 155 (78.3%) participants were female and 43 (21.7%) were male. Those with GO were older than GD (54.1 \pm 12.0SD years and 47.4 \pm 14.0SD years, respectively; $p < 0.001$). The median duration of Graves' disease diagnosis was 8 years in GO and 3 years in GD. There were more smokers and ex-cigarette smokers, non-metropolitan residents, radioactive iodine- and thyroidectomy-treatment in GO than GD (Table 1). GO had features of moderate to severe disease, 20 (19.8%) with optic neuropathy, 9 (8.9%) with exposure keratopathy, 84 (83.1%) required rehabilitative surgery for the complications of GO, and 76 (75.2%) had either a moderate or severe appearance. In total 26 cases had sight-threatening GO with either optic neuropathy, or exposure keratopathy, or both. There were 197 recordings of TSH, 190 recordings of fT4, 169 recordings of fT3 and 93 of TSH receptor antibodies. Median TSH level was significantly higher in GO (1.31 μ M/L{0.05,

3.21)) than GD (0.09uM/L{0.01,1.81}) (p<0.001). Median fT3 level was significantly lower in GO (4.5pmol/L{3.9, 5.3}) than GD (5.35pmol/L{4.3,9.85}) (p0.001). The median fT4 level was lower in GO (16.3pmol/L) than GD (17.2pmol/L) but was not statistically significant. (p 0.12) There was no significant difference in the median TSH receptor antibody levels in GO (1.58IU/L) and GD (1.10IU/L).

Mean serum selenium levels were significantly lower in GO (1.10+/-0.18 μ mol/L) compared with GD 1.19+/-0.20 μ mol/L (Figure 1). The difference in mean levels between cases and controls was 0.09 μ mol/L (95% CI{0.04, 0.14}, p 0.001). Mean selenium levels also appeared to decrease in parallel with increasing severity of GO; selenium level was 1.19 +/- 0.20 μ mol/L in GD, 1.10 +/-0.19 μ mol/L in moderate to severe GO, and 1.09 +/-0.17 μ mol/L in sight-threatening GO (p 0.003) (Figure 2).

In univariate analyses, the significant associations for GO were serum selenium (p<0.001), age (p<0.001), smoking status (p<0.001), TSH (p=0.007), fT4 (p 0.005), fT3 (0.001), ethnicity (p 0.025), residential location (p<0.001), duration of Graves' disease (p< 0.001), use of anti-thyroid medication (p=0.001), radioactive iodine treatment (p=0.001) and thyroidectomy (p<0.001). The association of lower serum selenium with GO did not differ when variables that were significant in univariate analysis were included in a multivariate model. Serum selenium levels remained significantly lower in GO after adjusting for age, smoking status, thyroidectomy, radioactive iodine and residential location (Table 2). The Graves' orbitopathy cases were 91% more likely to have lower selenium level than Graves' without orbitopathy. (Odds ratio{95% CI};0.09{0.01,0.67}, p=0.019)

Discussion

We found a small, but significantly lower serum selenium level in patients with moderate to severe Graves' orbitopathy (GO) compared with Graves' without orbitopathy (GD). There appeared to be a graded response with reducing selenium level in increasing severity of GO, the mean selenium difference between moderate severe GO subgroup and sight threatening GO subgroup was marginal. The lower serum selenium level in GO remained significant after adjusting for age, smoking status, radioactive iodine, thyroidectomy, and non-metropolitan location of residence. As expected, we found patients with GO were significantly older, a higher proportion were current or ex-smokers compared with controls. More GO patients required radioactive iodine and thyroidectomy treatment compared with controls. The differences in baseline features between our cases and controls were consistent with previous studies.¹²⁻¹⁴ Serum selenium was not associated with thyroid function in our study population.

In a previous study, selenium significantly improved quality of life, reduced progression of mild active thyroid orbitopathy and improved soft tissue changes, eyelid aperture and appearance in mild thyroid orbitopathy. This was demonstrated in a double-blind, randomised controlled trial of 150 patients with GO, treated with 100µg selenium twice a day for 6 months compared with either pentoxifylline 600mg twice a day, or placebo.¹⁰ This was the first randomized control study showing selenium supplementation is beneficial in GO. Trials on selenium supplementation in Graves' disease are scarce. A randomized controlled trial (GRASS trial) to determine the effect of selenium supplementation in Graves' disease was recently registered, and is currently recruiting.¹⁵ The supplementation of selenium for treating GO warrants further randomized controlled studies to validate the benefits in populations with differing baseline selenium status, and to determine the optimum dosage and selenium formulation. Selenium supplementation of 200 ug/day (as sodium selenite) over

6 months in GO was not associated with adverse events in a European population where marginal selenium deficiency was reported in the population.¹⁰

Our study is the first to show lower selenium level in patients with GO compared with GD in an Australian population with adequate selenium intake. Selenium intake is sourced from proteinaceous food such as fish, shellfish, meat, offal, cereals and grains, notably Australian wheat is rich in selenium.^{1, 16, 18} Levels that we found in Victorian blood donors are consistent with the marginally lower levels found in British people (1.1 $\mu\text{mol/L}$) than the higher levels found in North Americans (1.75 $\mu\text{mol/L}$).¹ Using a different technology selenium levels similar to our ranges were described in a South Australian study (1-1.6 $\mu\text{mol/L}$).¹⁹ This is different from another Australian study using 140 healthy subjects who found levels 0.8-2.2 $\mu\text{mol/L}$.²⁰ Mean selenium levels found in two different healthy Australian populations was 1.27 $\mu\text{mol/L}$ and 1.30 $\mu\text{mol/L}$.^{19, 20} There is no consensus for the use of serum selenium as the marker of selenium status, or a universal normal reference range values. At a serum selenium level of 100 $\mu\text{g/L}$ (1.27 $\mu\text{mol/L}$), which correlates with optimum glutathione peroxidase activity,²⁰ 76% of our study population fall below this level, that is 83 GO cases (83%) and 72 GD controls (72%). Serum selenium at 0.75 $\mu\text{mol/L}$ or below is considered deficient,²¹ 1% of GO and GD respectively of our study population were in this category. Two GD controls (2%) and 9 GO cases (9%) are below the 95% confidence interval lower limits of 0.83 $\mu\text{mol/L}$. Thus, marginal selenium status is prevalent in our study population. This may imply that even as the selenium level is only marginally lower in GO compared with GD, the capacity for handling increased oxidative stress in the orbit was compromised due to the presence of suboptimal selenium status.

In a Danish population, serum selenium levels in newly diagnosed Graves' disease were lower than in randomly selected normal controls (mean 1.14 $\mu\text{mol/L}$ versus 1.25 $\mu\text{mol/L}$,

p<0.01) and were also marginally lower in patients with autoimmune hypothyroidism than in normal controls, selenium level was not associated with the thyroid function status of the patients.² The study suggested a link between selenium deficiency and autoimmune thyroid disease.² In another study that compared serum selenium levels in 83 patients in remission or relapse of Graves' disease, there were no significant differences in the selenium levels between the two groups (mean 0.92 $\mu\text{mol/L}$, SD0.28 versus 0.91 $\mu\text{mol/L}$, SD0.21), however, the authors noted the highest level of selenium (>1.52 $\mu\text{mol/L}$) in patients with Graves' disease in remission, and postulated a positive effect of selenium in Graves' disease outcomes.²²

Improving selenium status may improve GO in a number of ways including reducing oxidative stress in GO, modulating immune response and T cell functions. GD and GO inflammatory processes are dominated by increased oxidative stress with increased production of free radical oxygen species and cytokines.^{23, 24} Correcting selenium deficiency or supplementing selenium in individuals with adequate selenium may reinforce the activity of glutathione peroxidases and thioredoxin reductases in cells, or enhance proliferation of activated T cells.

Evidence of increased oxidative stress in GO comes from both laboratory and clinical studies. Normal retro-ocular fibroblasts demonstrated no measurable oxygen free radicals whereas retro-ocular fibroblasts from GO showed spontaneous oxygen free radicals production and has significantly higher intracellular superoxide dismutase(SOD) activity in resting state, indicating the orbital fibroblasts is subjected to increased oxidative stress. Retro-orbital fibroblasts were less responsive to interleukin 1B stimulation in free radical generation and SOD induction.²⁶ Superoxide radicals induce a dose dependent orbital fibroblast proliferation in severe GO, not observed in fibroblast cultures of normal controls.²⁴ In patients with GO,

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reactive oxygen species decreased and antioxidants increased significantly in the blood with normalization of thyroid function. The antioxidants superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase levels in GO remained significantly reduced even after normalization of thyroid function compared with normal subjects.²⁷

Evidence of immune enhancement from selenium comes mainly from animal and *in vitro* studies. Selenium supplementation of 400 µg/day in healthy elderly significantly increased total T cell count by 27% more than placebo from an increase in CD4+ T cells and enhanced natural killer cell activity.²⁸ UK adults with low selenium status receiving 50 µg and 100 µg per day of sodium selenite was also noted to clear challenge dose of live attenuated poliovirus more readily than those given placebo.²⁹ Selenium supplements augmented cellular immunity by increased production of interferon gamma and other cytokines, an earlier peak of T cell proliferation and an increase in T helper cells.²⁹ Selenium supplementation also correlates with lymphocyte proliferation and increased expression of interleukin-2 receptor in humans.¹ Selenium promotes T helper type 1 (Th1) differentiation i.e. cellular immunity and diverts away from T helper type 2 (Th2) differentiation i.e. humoral immunity.²⁵

The role of selenium in changing the Th1/Th2 balance and its role in T cell activation in GO will require further studies. Perpetuation of GO orbital inflammation results from infiltration of activated T cells into the orbit. Th1 cells and cytokines predominate early in thyroid orbitopathy whereas Th2 cells and cytokines predominate later in its course.³⁰ One study on the peripheral blood T cell profile in GO and GD showed a shift towards Th1 dominance in GO compared with GD and healthy controls, with a higher ratio of CD8-/IFNgamma+ to CD8-/IL4+ T cells (Th1/Th2) and a predominance of CD4+ T cells.³¹

Our study has some limitations, Selenium levels in serum were only marginally lower in GO versus GD, with both groups well within the 95% reference intervals. The physiological

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significance of the small mean selenium difference between GO and GD remains uncertain, as plasma selenium does not represent tissue selenium levels and the absolute measurement might underestimate local selenium requirements. The association of non-metropolitan location with Graves' orbitopathy and lower selenium levels in our view should not be over-interpreted as there was only one (1%) GD control from non-metropolitan area and the odds ratio for Graves' orbitopathy from non-metropolitan area has a wide 95% confidence interval in multivariate analyses. There are also a number of missing values in fT3 and TSH receptor antibody levels as they were not routinely measured in our study, hence analyses pertaining to thyroid hormone and TSH receptor antibody measurements were limited.

In conclusion, we found a small, but significant, difference in selenium levels, being lower in GO than GD in a study population with marginal selenium status, not accounted for by differences in age, smoking status, residential location, anti-thyroid drug treatment or thyroidectomy. We conclude relative selenium deficiency may be an independent risk factor for orbitopathy in patients with Graves' disease.

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Table 1

Baseline demographics of Graves' orbitopathy versus Graves' control, variables having significant association with Graves' orbitopathy with $p < 0.05$ ++

| Variables | Graves' Orbitopathy (GO) | Graves' orbitopathy (GD) | All | P value |
|---------------------|--------------------------|--------------------------|-----|---------|
| N | n=101 (51%) | n=97 (49%) | 198 | |
| Mean age | 54.1 +/-12 | 47.4 +/-14 | | <0.001 |
| Mean serum selenium | 1.10 +/- 0.18 umol/L | 1.19 +/- 0.20 umol/L | | 0.001 |

| | | | | | |
|--------------------------|---------------------|------------------------|------------------------|-----|--------|
| Sex | Female | 75 (74%) | 80 (82%) | 155 | 0.161 |
| | Male | 26 (26%) | 17 (18%) | 43 | |
| Smoking status | Non smoker | 26 (26%) | 54 (56%) | 80 | <0.001 |
| | Cigarette Smoker | 27 (28%) | 14 (14%) | 41 | |
| | Ex-cigarette smoker | 45 (46%) | 29 (30%) | 74 | |
| Caucasian | | 93 (92%) | 79 (81%) | 172 | 0.027 |
| Metropolitan | | 78 (77%) | 96 (99%) | 174 | <0.001 |
| Non metropolitan | | 23 (23%) | 1 (1%) | 24 | |
| Anti-thyroid medications | | 87 (86%) | 95 (98%) | 182 | 0.001 |
| Radio-active iodine | | 36 (36%) | 15 (15%) | 51 | 0.001 |
| Thyroidectomy | | 36 (36%) | 6 (6%) | 42 | <0.001 |
| TSH | | 1.31 uM/L (0.05, 3.21) | 0.09 uM/L (0.01, 1.81) | 197 | <0.001 |
| T3 | | 4.5 pmol/L (3.9, 5.3) | 5.35 pmol/L (4.3, 985) | 169 | 0.001 |

++parametric data refers to mean +/-SD, non parametric data refers to median + interquartile

range *Missing value smoking status in 3 Graves' orbitopathy cases

Table 2

Final multiple logistic regression model including variables having significant associations with Graves' orbitopathy versus controls.

| Predictor | Coefficient | SE Coef | z | P value | Odd Ratio | 95% CI for OR |
|--------------------|-------------|---------|-------|---------|-----------|---------------|
| Selenium level | -2.45 | 1.05 | -2.34 | 0.019 | 0.09 | 0.01,0.67 |
| Age | 0.046 | 0.015 | 3.00 | 0.003 | 1.05 | 1.02,1.08 |
| Current Smoker | 1.77 | 0.55 | 3.21 | 0.001 | 5.85 | 1.99,17.22 |
| Ex-smoker | 1.44 | 0.44 | 3.27 | 0.001 | 4.24 | 1.78,10.09 |
| Radioactive iodine | 1.16 | 0.45 | 2.61 | 0.009 | 3.20 | 1.34,7.65 |
| Thyroidectomy | 2.52 | 0.54 | 4.64 | <0.001 | 12.41 | 4.29,35.92 |
| Non metropolitan | 2.94 | 1.08 | 2.72 | 0.006 | 18.98 | 2.28,158.00 |

Figure 1

Individual value dot plot showing lower mean serum selenium level in patients with moderate to severe Graves' orbitopathy (Group1) compared to patients without Graves' orbitopathy (Group 0) The normal Victorian adult selenium level reference range is 0.8-1.4umol/L

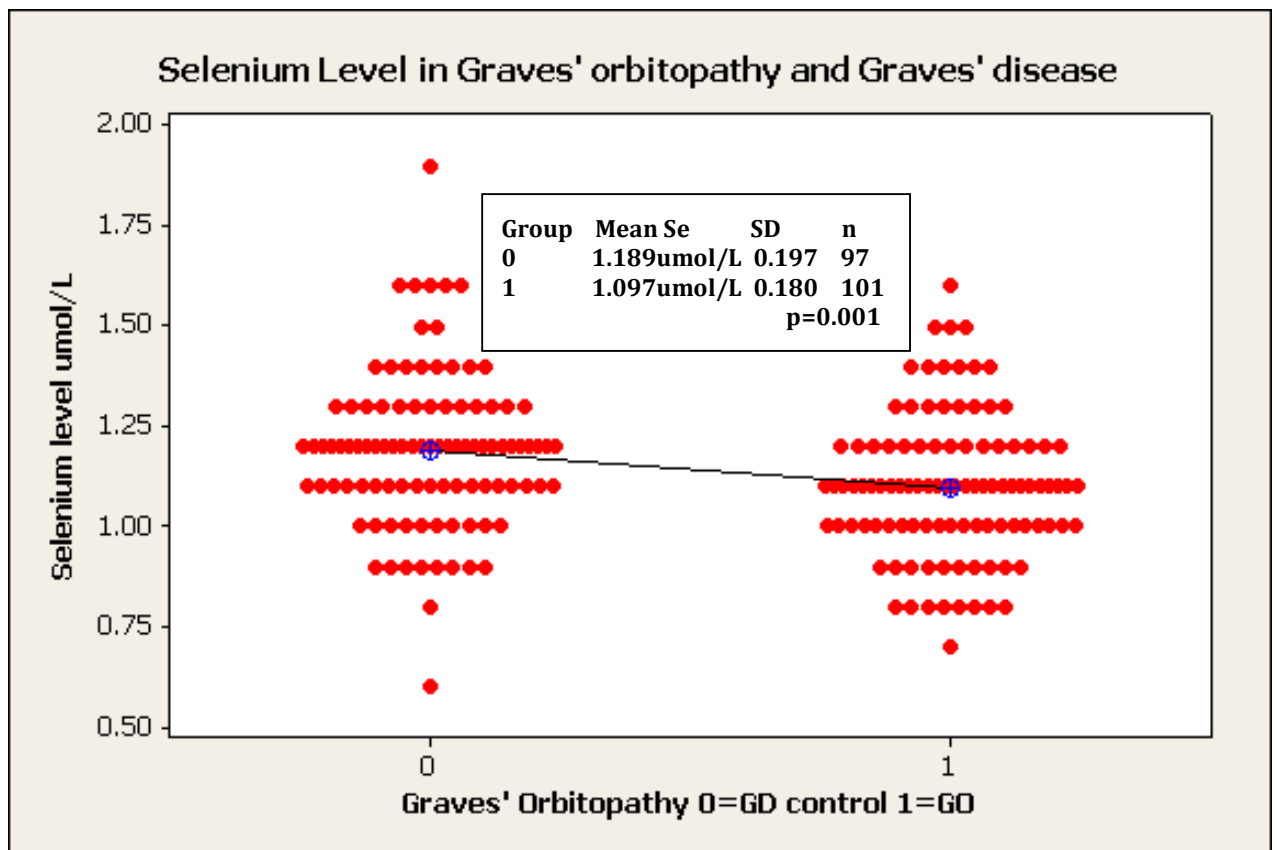


Figure 2

Mean selenium levels and 95% confidence intervals for Graves' without orbitopathy, moderate to severe Graves' orbitopathy and sight threatening Graves' orbitopathy, One-way Anova $p=0.003$

