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MELATONIN IN CHAGAS´ DISEASE. POSSIBLE THERAPEUTIC VALUE

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Abstract Chagas' disease is a severe health problem in Latin America, causing approximately 50 000 deaths a year, with approximately 18 million infected people. About 25-30% of the patients infected with *Trypanosoma cruzi* develop the chronic form of the disease. The protective response against *T. cruzi* depends on both innate and acquired immunity involving macrophages, natural killer cells, T and B lymphocytes, and the production of proinflammatory Th-1 cytokines. In addition, an increased nitric oxide (NO) production in macrophages leading to effective microbicidal action is needed to control parasitemia. Melatonin is detectable in *T. cruzi* and may play a role in promoting infection whereas, when administered in high doses during the acute phase of *T. cruzi* infection, it can decrease parasitemia while reducing NO production. During chronic disease progression, the sustained oxidative stress concomitant to myocardial damage could be reduced by administering melatonin. It is hypothesized that the coordinated administration of a melatonin agonist like the MT_1/MT_2 agonist ramelteon, that lacks antioxidant activity and may not affect NO production during the acute phase, and of melatonin in doses high enough to decrease oxidative damage, to preserve mitochondrial and to prevent cardiomyopathy during the chronic phase, could be a novel add-on treatment of Chagas´ disease.

Key words: Chagas´ disease, melatonin, melatonin receptors, oxidative stress, nitric oxide

Resumen ***La melatonina en la enfermedad de Chagas. Su posible valor terapéutico.*** La enfermedad de Chagas es un problema grave de salud en América Latina, causando cerca de 50 000 muertes al año y unos 18 millones de infectados. Alrededor del 25-30% de los pacientes infectados con *Trypanosoma cruzi* desarrollan la forma crónica de la enfermedad. La respuesta de defensa ante el *T. cruzi* depende de la inmunidad innata y adquirida con la participación de macrófagos, células "natural killer", linfocitos T y B, y la producción de citoquinas proinflamatorias de tipo Th-1. Además, el aumento en la producción de óxido nítrico (NO) en los macrófagos lleva a una acción microbicida eficaz necesaria para controlar la parasitemia. La melatonina es detectable en *T. cruzi* y podría desempeñar un papel en la promoción de la infección como lo hace en el paludismo, mientras que, cuando se administra en dosis farmacológicas altas durante la fase aguda de la infección por *T. cruzi*, disminuye la parasitemia, aun en presencia de una reducción de la producción de NO. Durante la progresión de la enfermedad de Chagas a la cronicidad, el estrés oxidativo aumentado con el concomitante daño miocárdico podría reducirse por la administración de melatonina, de reconocida acción antioxidante. Se propone como un nuevo enfoque complementario en el tratamiento de la enfermedad de Chagas la administración durante la fase aguda de un agonista MT1/MT2 de la melatonina como el ramelteon, que carece de actividad antioxidante y podría no afectar a la producción de NO, y de melatonina durante la fase crónica de en dosis suficientemente altas como para disminuir el daño oxidativo y prevenir la miocardiopatía.

Palabras clave: enfermedad de Chagas, melatonina, receptores melatoninérgicos, estrés oxidativo, óxido nítrico

The ubiquitous methoxyindole melatonin is secreted by the pineal gland of most mammals, including man and additionally, its presence has been confirmed in many plants and unicellular organisms[1] including *Trypanosoma cruzi*[2]. Melatonin participates in diverse functions of the body including sleep and circadian rhythm regulation, immunoregulation and free radical scavenging, and may have anti-cancer actions (for ref. see[3]). Melatonin also protects organisms against bacterial, viral and parasitic infections by a variety of mechanisms and has been shown to be beneficial for reversing symptoms of septic shock[4].

Chagas´ disease is a frequent anthropozoonosis in Latin America caused by *T. cruzi*, a parasitic protozoan of the ancient branch of eukaryotes. Chagas' disease affects 16-18 million people from Southern California to Argentina and Chile with nearly 100 million people at risk of infection[5-7]. It is a major public health concern in Latin America with around 50 000 deaths per year taking second place after malaria in prevalence and mortality due to vector associated diseases.

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Transmission of *T. cruzi* occurs predominantly via insect vectors of the subfamily *Triatoma* that reside in the peridomestic habitat of mud-thatch houses in rural areas. Blood transfusion and organ transplantation represent further routes of *T. cruzi* transmission. Although the pathophysiology of Chagas' disease is not completely understood, it is widely accepted that the involvement of the immune response is critical in determining the outcome of the disease[8,9]. The protective response against *T. cruzi* depends both on innate and acquired immunity involving macrophages, natural killer (NK) cells, T and B lymphocytes, and the production of many different cytokines, which play key roles in regulating both parasite replication and the immune response[10].

The innate immune response to *T. cruzi* involves production of cytokines that modulate cell activity, including interferons (IFN) and interleukin (IL)-12. IFN-γ augments nitric oxide (NO) production in macrophages leading to effective microbicidal action. In acute infection some parasite antigens can activate macrophages, and this may result in pro-inflammatory cytokine production, nitric oxide (NO) synthesis, and consequent control of parasitemia and mortality. Some trypanocidal drugs such as nifurtimox and benznidazole act by enhancing radical oxygen species (ROS) generation during their metabolism[11,12]. *T. cruzi* is very susceptible to the cell damage induced by these metabolites because enzymes scavenging free radicals are absent or have very low activities in the parasite. Cell-mediated immunity in *T. cruzi* infection is also modulated by cytokines and autoimmunity can be triggered[8,9].

Despite this strong anti-parasite immune response, *T. cruzi* persists in a majority of hosts, the causes for immune evasion and persistence being largely unknown. Infection by *T. cruzi* elicits both humoral and cell-mediated immunity directed by a T-helper (Th) type 1 cytokine response. $CD8^+$ T cells are essential for the control of the intracellular amastigote stages via IFN-γ secretion[8,9].

Melatonin is detectable in *T. cruzi* where it seems to promote parasitemia[2] but when administered during the acute phase of *T. cruzi* infection it reportedly reduces parasitemia[13-15]. The activity of melatonin during the acute phase may be partly related to the well-demonstrated immunoregulatory role the methoxyindole has[3]. However, melatonin decreases NO production by macrophages and may harm their effective trypanocidal action during the acute phase of the disease[16]. During the chronic phase, treatment with melatonin could be beneficial for combating the sustained oxidative stress concomitant to imyocardial damage[17], therefore impairing disease progression.

Melatonin, the immune system and ROS generation

Melatonin is the major neurohormone secreted by the pineal gland during the dark hours at night. In addition, melatonin has been demonstrated to be produced by many organs other than the pineal gland, including the retina, the gastrointestinal tract, skin, leukocytes, the thymus and bone marrow cells[3]. The role of melatonin in the immune system and as far as ROS generation has been reviewed several times. The intention of this section is not to summarize in detail all of the knowledge in this field, but rather to highlight several facts which are of importance for the discussion of its potential relevance in Chagas´ disease.

Melatonin is formed by various leukocytes, including monocytes, eosinophiles, mast cells, T-lymphocytes, NK cells, bone marrow cells and several leukocyte-derived cell lines[3]. Melatonin biosynthesis has been also described in thymocytes and epithelial cells. The simultaneous biosynthesis of melatonin and expression of melatonergic MT_1 receptors in many of these cells[18] not only indicate that the methoxyindole has a role in communication within the immune system, but also possesses autocrine, paracrine and, perhaps, intracrine functions.

Besides binding to MT_1 and MT_2 membrane melatonin receptors[19], melatonin also binds to transcription factors belonging to the retinoic acid receptor superfamily, in particular, splice variants of RORα and the product of another gene, RZRβ[3]. The simultaneous expression of membrane receptors and RORα subforms in various leukocytes seems to be of particular relevance for local actions, which might include a focal or intracellular accumulation of melatonin. The reported positive correlation between MT_1 signaling and RORα expression in leukocytes[18] indicates a concerted action of these two types of receptors. It has been concluded that melatonin through RORα binding, stimulates the secretion of IL-2, IL-6 and IFN-γ by T-helper cells type 1 and by monocytes[20].

The functional spectrum of immunomodulation by melatonin is highly complex and involves various cytokines. The main findings on this point comprise stimulation of IL-2, Il-6 and IFN-γ formation in T-helper cells and monocytes, counteraction of the inhibitory effect of prostaglandin E_2 (PGE_2) on IL-2 production, and secretion of IL-1, IL-12, tumor necrosis factor (TNF)-α and macrophage-colony stimulating factor (M-CSF) in monocytes and/or monocyte-derived cells[3]. These effects are not only related to cytotoxicity, prooxidant and pro-inflammatory actions, but also to differentiation and interactions of T-lymphocytes with antigen-presenting (AP) cells. Melatonin promotes the expression of MHC class II molecules and transforming growth factor (TGF)-β in AP cells and influences, via IL-12, T-cell differentiation and growth in favor of Th-1. In addition to Th-1activation, melatonin has also been reported to promote Th-2 responses, findings that may require further substantiation and mechanistic explanation, since, under other conditions, such as trypanosome infection, melatonin was found to decrease Th-2 responses[15].

The pro- and anti-inflammatory actions of melatonin deserve particular attention[3]. At first glance, these effects appear to be contradictory. However, these actions have to be interpreted in the context of different conditions. As far as it enhances the immune response via AP cells and T-lymphocytes, as well as the activation of monocytes and other ROS-generating cells by the methoxyindole alone, melatonin behaves in a prooxidant way. Under other conditions, however, melatonin can reduce oxidant formation, which has been repeatedly shown in numerous experiments using bacterial lipopolysaccharide (LPS)[4]. In particular, TNF-α was down-regulated. Moreover, melatonin inhibits the LPS-induced expression of various chemokines in peripheral blood mononuclear cells.

The presence of melatonin as well as MT_1 and MT_2 in a mast cell line was taken as evidence implicating the methoxyindole´s modulatory effects in these cells, too[21], which might consist in an anti-inflammatory action via inhibition of TNF-α release. Other anti-inflammatory actions of melatonin, in addition to down-regulation of 5-lipoxygenase, concern to the antagonism to PGE_2 and the inhibitory effects on PG synthesis[22]. Melatonin as well as its metabolites N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK) were shown to down-regulate cyclooxygenase expression in macrophages[23].

One of the strongest anti-inflammatory effects of melatonin concerns the down regulation and inhibition of inducible and neuronal NO synthases (iNOS and nNOS)[3]. It should be stressed that melatonin does not substantially interfere with basal or moderately elevated NO levels and that melatonin and also AMK are highly effective in protecting from NO-mediated mitochondrial blockades and cell damage under severe inflammatory conditions such as sepsis[4].

Because ROS generation is a continuous and physiological phenomenon, cells possess efficient antioxidant systems that protect them from oxidative damage. Data accumulated in the last decade strongly indicate that melatonin plays an important role in this defense[24]. Because of its amphiphilic properties melatonin passes through all biologic barriers with ease. Melatonin gets access freely to all compartments of the cell, and is especially concentrated in the nucleus and mitochondria[25].

The discovery that melatonin was a remarkably potent scavenger of the particularly reactive, mutagenic and carcinogenic hydroxyl radical (•OH)[26] was the finding that initiated numerous studies on melatonin´s role as a protector against free radicals. Melatonin reacts mainly with superoxide anion radical (O_2•$^-$), hydrogen peroxide (H_2O_2), •OH and nitric oxide radical (•NO) and its derivative peroxynitrite ($ONOO^-$). Although direct radical scavenging has been effective under numerous experimental conditions at clearly supraphysiological melatonin concentrations, its relevance at physiological levels has been questioned for reasons of stoichiometry[3]. Even though a single melatonin molecule may generate products in a scavenger cascade which may collectively eliminate up to ten free radicals, such findings from chemical systems may not be fully applicable to physiological conditions.

In spite of this, melatonin was shown to protect from oxidotoxicity already at physiological concentrations[27]. A possible indirect action as mediated by up-regulation of antioxidant enzymes by melatonin was proposed. More recently, a number of mitochondrial effects of melatonin have come into focus, including safeguarding of respiratory electron flux, reduction of oxidant formation by lowering electron leakage) and inhibition of opening of the mitochondrial permeability transition pore[3,28]. These effects of melatonin and its metabolites are rather unique. For example, the MT_1 / MT_2 melatonergic agonist ramelteon displays no relevant antioxidant activity[29].

Melatonin and Chagas´ disease

Infection by *T. cruzi* elicits acute non-specific symptoms e.g. fever, malaise, edema and/or enlarged liver or spleen to the characteristic swelling at the site of entry. In spite of the high blood parasitemia in the acute phase, clinical symptoms do not warranty hospital visit, and therefore, anti-parasitic treatment is often not initiated. In few (<5%) acute patients, sudden death due to congestive heart failure associated with myocarditis or meningoencephalitis may occur[30].

Most individuals survive the acute infection and enter an “indeterminate” phase that is defined by the presence of *T. cruzi* specific antibodies but the absence of clinical signs of cardiac abnormalities[31]. Approximately 10–20 years after the initial infection, and depending on the region, about 30% of those infected manifest severe cardiomyopathy or gastrointestinal dysfunction. The clinical manifestations of chagasic cardiomyopathy comprise congestive heart failure, thromboembolism in brain, limbs or lungs, and ventricular fibrillation[32-34].

T cruzi multiplies as flagellate epimastigote forms in the gut of the hematophagus insect with a subsequent transformation in the metacyclic trypomastigotic infective forms in the rectum of the insect host[35]. This last process exhibits a 24-h periodicity, mainly occurring under darkness. Endogenous melatonin in the parasite may play a promoting role in this phenomenon[2]. When exposing the epimastigote forms of *T. cruzi* to a LD cycle of 0:24 h, the parasite reached exponential growth by the 7th day. A pulse of 2 h of light (LD 2:22) was enough to block growth of the epimastigotes[2], an effect that was correlated with the expression of heat-shock proteins and inhibition of parasitisation capacity. Epimastigotes cultured under continuous darkness produce melatonin over the 24 h period while under a LD cycle of 2:22 h showed a 55% decrease. The incubation of epimastigotes with melatonin (1 pM)

did not affect parasite growth, but significantly reduced their transformation into metacyclic forms. The authors concluded that the light-dependent decrease in melatonin production by the unicell was responsible, at least partially, for the light-induced parasitisation inhibition[2].

From the insect dejections, the trypomastigote forms of *T. cruzi* are able to enter the skin nucleate cells of the vertebrate host, where they become amastigotic forms. After multiplication in the cytoplasm, amastigotes become trypomastigotes that escape from the host and invade other cells. The vectors acquire the parasite after feeding on blood from an infected animal[35].

It is clear that a *T. cruzi* acute infection like other parasitic diseases is a serious challenge for immune host defense mechanisms before, during and after entry into host´s cells. Multiple components of both the innate and the adaptive immune systems are simultaneously required for protection during the acute phase of infection, with IFN- induced NO production being an important mediator of resistance to *T. cruzi*[10].

The first demonstration that melatonin treatment can control *T. cruzi* infection during the acute phase was given by Santello and co-workers in *T. cruzi*-infected male Wistar rats[13]. Rats received 5 mg of melatonin/kg p.o. daily either beginning 7 days prior to infection or concomitantly with the infection. Rats treated with melatonin showed a significant reduction in the number of blood trypomastigotes and an increased number of leucocytes as compared to untreated animals. This effect coincided with a significant increase in IL-2 levels. Melatonin administration also resulted in fewer and smaller amastigote burdens, and less inflammatory infiltrate and tissue disorganization in the heart[13].

Other studies from the same group of investigators and using a similar experimental design ensued. They reported that 5 mg/kg of melatonin p.o. daily either beginning 7 days prior to *T. cruzi* infection or concomitant with the infection augmented the circulating levels of IL-12, IFN- and TNF-[14], and decreased the circulating levels of IL-4, IL-10 and tumor growth factor-[15], in particular after concomitant administration with the parasites. Melatonin treatment reduced the levels of NO, increased the number of peritoneal macrophages and impaired splenocyte proliferation after concanavalin A exposure. The authors concluded that the effect of melatonin on *T. cruzi* infection was exerted via enhanced Th-1 cytokine production[14] and suppressed Th-2 response[15] consequently promoting a reduction in blood and tissue parasites[13].

Lately, the possible synergism between melatonin and the cyclooxygenase inhibitor meloxicam in up-regulating the immune response in male Wistar rats infected with *T. cruzi* was tested[36]. The combined treatment with melatonin and meloxicam significantly enhanced the release of IL-2 and IFN- into animals' serum, when compared with the infected control groups during the course of infection. The blockade of prostaglandin E_2 synthesis and the increased release of NO by macrophage cells from *T. cruzi*-infected animals contributed to regulate the production of Th-1 subset cytokines significantly reducing the parasitemia in animals treated with the combination of both substances[36].

ROS and oxidative stress have been hypothesized to play major roles in the development of systemic complications of Chagas´ disease and in particular Chagas´ cardiomyopathy[17,37-40]. Several studies in animals have shown that a cycle of mitochondrial functional decline and ROS generation, coupled with an inability to efficiently scavenge the mitochondrial ROS, predispose the chagasic hearts to sustained oxidative stress during infection and disease development. This correlates with clinical findings indicating a general increase of oxidative stress parallel to the progression of Chagas´ disease[17,39].

As recently reviewed[41,42], melatonin has a number of attributes predisposing it to the protection of the hypertrophied heart. In general, the effects of melatonin on the heart and blood vessels are beneficial and improve the physiology of the circulatory system. Melatonin is effective in protecting the cardiac musculature against ischemia-reperfusion[43-45] and effect attributed to its action in inhibiting the mitochondrial permeability transition pore and in preserving the content and integrity of cardiolipin molecules[3]. Melatonin treatment resulted in significant reductions in infarct size[46,47]. Melatonin interferes with the central and peripheral vegetative nervous system and supports the dominance of the cholinergic over the adrenergic system. Therefore, the use of melatonin to delay the onset of oxidative insult and mitochondrial deficiency in the myocardium, would prove to be effective in preserving cardiac functions in Chagas´ disease. For example, in malarial infection the development of mitochondrial pathology and mitochondrial oxidative stress in hepatocytes was effectively prevented by melatonin[48].

In conclusion the studies discussed above underline the complexity of melatonin's effects on Chagas´ disease (Fig. 1). During the acute phase, melatonin administration may have both enhancing and inhibitory effects on *T. cruzi* development. Through receptor-mediated promotion of Th-1 inflammatory response melatonin can be helpful to decrease parasitemia after *T. cruzi* infection. However, Inhibition of NO production, presumably a non-receptor mediated effect of melatonin, may harm the optimal phagocytic activity of macrophages, like that needed in the acute phase of Chagas´ disease. The use of MT_1/MT_2 melatonin agonists such as ramelteon during the acute phase could be appropriate since ramelteon displays no relevant antioxidant activity as compared to melatonin[29]. On the other hand, during the indeterminate phase, the administration of melatonin to inhibit free radical-mediated mitochondrial impairment could be useful to limit ROS production and ROS-induced cardiomyopathy (Fig. 1).

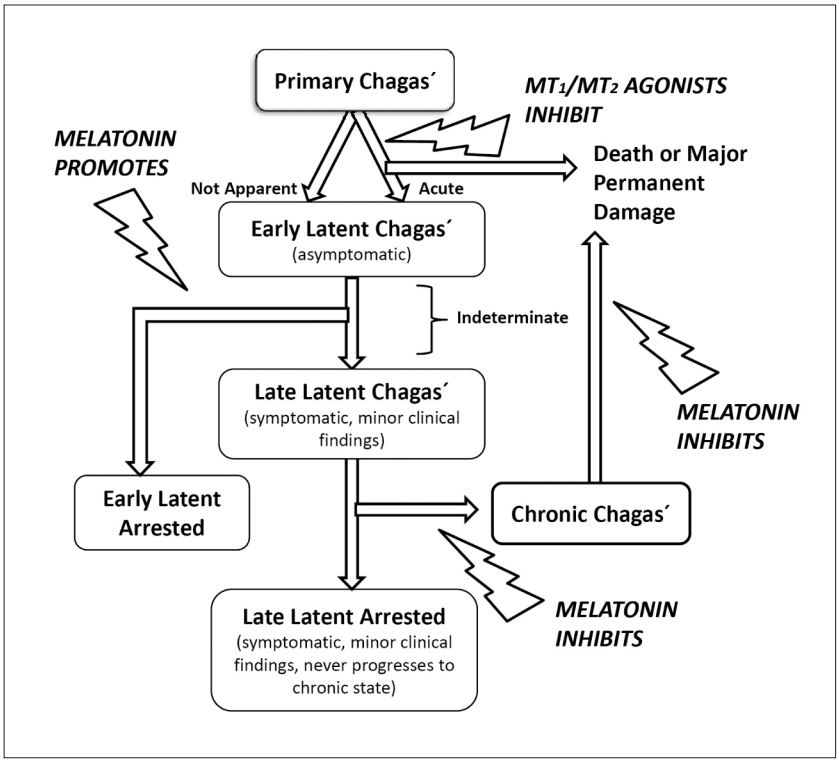

Fig. 1.– Scheme summarizing the possible therapeutical validity of melatonin and melatonin analogs in Chagas´ disease. It is hypothesized that the administration of melatonin agonists could promote immunity without impairing NO production during the acute phase and that exogenous melatonin administration during the indeterminate phase could inhibit free radical-mediated mitochondrial-dependent apoptosis and cardiac damage to prevent chronic Chagas´ disease

It is therefore hypothesized that the coordinated administration of a melatonin agonist like the MT_1/MT_2 agonist ramelteon, that may promote immunity without impairing NO production during the acute phase, and of melatonin in doses high enough to decrease oxidative damage, to preserve mitochondrial and to prevent cardiomyopathy during the chronic phase, could be a novel add-on treatment of Chagas´ disease (Fig. 1). This can be explored in animals and eventually in humans in view of very low toxicity melatonin displays, as indicated by toxicological studies in rodents[49] and clinical studies in children and adult humans[50-53]. Chronic treatment with melatonin has the additional advantage of its low cost, an ideal condition to treat an "orphan disease" like Chagas´ disease, which is far more prevalent in developing countries than in the developed world.

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Of course, everyone knows that failure to reject the Fisherian null hypothesis does not warrant the conclusion that it is true. Fisher certainly knew and emphasized it, and our textbooks duly so instruct us.

Yet, how often do we read in the discussion and conclusions of articles now appearing in our most prestigious journals that "there is no difference" or "no relationship"?

The other side of this coin is the interpretation that accompanies results that surmount the .05 barrier and achieve the state of grace of "statistical significance" ."Everyone" knows that all this means is that the effect is not nil, and nothing more. Yet how often do we see such a result to be taken to mean, at least implicitly, that the effect is significant, that is, important, large. If a result is highly significant, say p <.001, the temptation to make this misinterpretation becomes all but irresistible. […]

There is no ontological basis for dichotomous decision making in psychological inquiry. The point was neatly made by Rosnow and Rosenthal (1989) in the American Psychologist . They wrote "surely, God loves the .06 nearly as much as the .05" (p. 1277). To which I say amen!

Por supuesto, todo el mundo sabe que el fracaso en rechazar la hipótesis nula de Fisher no garantiza que la conclusión sea verdadera. Fisher ciertamente sabía y resaltaba esto, y nuestros libros de texto nos instruyen debidamente.

Aun así, ¿cuán a menudo leemos en la discusión y conclusiones de artículos que aparecen en nuestros más prestigiosos *journals*, que " no hay diferencia" o que "no existe relación"?

El otro lado de esta moneda es la interpretación de los resultados que sobrepasan la barrera del 0.05 y alcanzan el estado de gracia de "significación estadística". "Cualquiera" sabe que ello significa que el efecto no es nulo, y nada más. Sin embargo, cuán a menudo vemos tal resultado tomado queriendo decir, al menos en forma implícita, que el efecto es *significativo*, es decir *importante, sustantivo*. Si un resultado es altamente significativo, tal como p <0.001, la tentación de llegar a esta equivocada interpretación, se hace prácticamente irresistible.

No existen bases ontológicas para tomar decisiones dicotómicas en las investigaciones psicológicas. Este punto fue claramente definido por Rosnow y Rosenthal (1989) en el *American Psycologist*. Ellos escribieron: "seguramente Dios ama casi tanto al 0.06 como al 0.05" (p 1277). A lo que yo digo amen!

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Things I Have Learned (So Far). *American Psychologist*, 1990; 45:1304-12

http://www.uvm.edu/~bbeckage/Teaching/DataAnalysis/AssignedPapers/Cohen_1990.pdf