

## Interaction between caffeine intake and heart zinc concentrations in the rat

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The purpose of the present study was to determine the levels of zinc in the hearts of growing post-weaning offspring, fetuses and their dams chronically fed caffeine. A further study was conducted to determine the distribution of Zn in subcellular heart fractions affected by acutely injecting caffeine into the veins of the adult rats. After delivery pups were raised on a 200 g protein/kg diet until day 22 of weaning. On day 22 randomly selected male offspring from each litter were divided into two groups. Group 1 was fed continuously on the same diet as a control, whereas in the experimental group offspring were fed on a 200 g protein/kg diet supplemented with caffeine (20 mg/kg). On day 49 the animals were killed and Zn, calcium and magnesium concentrations of the hearts were measured. In the second series of studies pregnant dams were randomly divided into two groups. Group 1 was fed on a 200 g protein/kg diet from day 3 of gestation, whereas in the experimental group dams were fed on the diet supplemented with caffeine. On day 22 of gestation the fetuses were surgically removed. The Zn, Ca and Mg concentrations of hearts of fetuses and dams were determined. In the third phase a caffeine solution was injected into the vein. After 45 min the hearts were removed and Zn levels in the subcellular fractions determined. The hearts of the growing offspring fed on a caffeine-supplemented diet consistently showed decreased Zn and Ca levels compared with the non-caffeine group. In contrast, Zn and Ca concentrations of the hearts of fetuses and dams showed no difference between caffeine and non-caffeine groups. In the various fractions studied, the Zn contents affected by caffeine were in the supernatant (cytoplasmic) fraction. This fraction contained 30% of the Zn concentration of the homogenates. Current findings suggest that caffeine intake and heart Zn levels are closely correlated.

### Caffeine: Heart: Zinc: Rat

Chronic caffeine consumption during early growth and development has been shown to affect cardiac performance in rats. Using an isolated heart preparation, the hearts of caffeine-fed rats over a period of 88 d after birth exhibited a significant reduction in cardiac output, stroke volume, mean aortic pressure and estimated myocardial work when compared with the non-caffeine group (Temples *et al.* 1985). Even though the caffeine feeding period was shortened to 50 d after birth, various factors measured showed the same impairment of function (Temples *et al.* 1987). Chronic caffeine ingestion has been shown to exacerbate renovascular hypertension (Ohnishi *et al.* 1986) and results in a moderate to severe myocardial fibrosis (Johansson, 1981). The mechanism which produces permanently altered cardiovascular function in the growing heart as a result of chronic feeding of caffeine is not known at the present time.

A previous study indicated that when caffeine is injected into the femoral vein in adult rats, Zn levels of the heart are decreased within a very short period of time (Rossowska & Nakamoto, 1990). However, Zn concentration also recovers quickly to normal levels. Zn is essential to growth (Dreosti, 1982) and plays an important role in maintaining membrane

structure and function (Bettger & O'Dell, 1981). Consequently, reduced tissue Zn levels as a result of chronic caffeine feeding during the rapid growth period in the developing heart might permanently affect its development. This could result in an impairment in cardiovascular function. Thus, the present studies were conducted to determine whether heart Zn levels are reduced by chronic caffeine feeding in growing rats, and in growing fetal rats and their dams. We have also determined the distribution of Zn in heart subcellular fractions in acute caffeine injected adult rats.

#### MATERIALS AND METHODS

##### *Effects of caffeine intake on post-weaning rats*

A total of ten timed-pregnant rats were purchased from the breeder (Holtzman Co., Madison, WI). They were maintained on a 12 h light–12 h dark cycle and fed on laboratory chow and water *ad lib*. At term, litters delivered within an 8 h period were combined and designated day 1; eight pups were randomly assigned to each dam. They were fed on a 200 g protein/kg diet (Temples *et al.* 1985) *ad lib*. Dietary composition has been described in detail (Harvey & Nakamoto, 1988). Calculated concentrations of Zn, Ca and Mg were 12, 5000 and 400 mg/kg diet respectively. When the offspring were weaned at day 22, a male pup from each dam was randomly selected and fed continuously on the same 200 g protein/kg diet as a control (*n* 10), whereas in the experimental group (*n* 10) pups were selected in the same way but fed on a 200 g protein/kg diet supplemented with caffeine (20 mg/kg body-weight). Caffeine levels in the diet were adjusted for increased body-weight and food intake as described by Nakamoto & Shaye (1986). Fresh diet was made every week. All pups were housed individually and water was provided *ad lib*. On day 49, pups were fasted for 3–4 h and were killed by decapitation; and the hearts were removed, blotted and weighed. All experiments were conducted between 10.00 and 12.00 hours. Each heart was homogenized in 2.5 ml double-distilled deionized water (DDDW) in the cold (4°) with a polytron homogenizing apparatus (Model No. SDT; Tekmar Co., Cincinnati, OH). Zn, Ca and Mg contents were measured using atomic absorption flame emission spectrophotometry (Model 280; Fisher Scientific Co., Fairlawn, NJ).

##### *Effects of caffeine intake during pregnancy on dams and fetuses*

Timed-pregnant dams were purchased from the same breeder. Starting on day 3 of gestation (sperm-positive day counted as day 1), the control dams were fed on a 200 g protein/kg diet and dams in the experimental group were fed on a 200 g protein/kg diet supplemented with caffeine (20 mg/kg body-weight). The amount of caffeine added to the maternal diet was calculated based on the average body-weight and mean daily food intake of the dams. Fresh diet was made every 5 d. On day 22 of gestation, the dams were anaesthetized with diethyl ether and killed by cervical dislocation. All fetuses were then delivered surgically. They were blotted and decapitated. The hearts were removed, blotted and four hearts of the litter were pooled and weighed. A set of four pooled hearts was homogenized with 2.5 ml DDDW using a polytron homogenizer. Hearts from each dam were also homogenized with 2.5 ml DDDW. Zn, Ca and Mg contents were measured using atomic absorption flame emission spectrophotometry, according to the procedure of Rossowska & Nakamoto (1990).

##### *Effects of caffeine on Zn distribution*

Adult female Sprague-Dawley rats which weighed 230–250 g were purchased from the same breeder. The animals were fed on the same laboratory chow as described previously (Rossowska & Nakamoto, 1990). They were anaesthetized with pentobarbital (60 mg/kg body-weight) by the intraperitoneal route, and the femoral vein was exposed for

Table 1. Zinc, calcium and magnesium concentrations in hearts of 49-d-old rats fed on 200 g protein/kg diet without or with 20 mg caffeine/kg body-weight from 22 to 49 d of age (Mean values with their standard errors; no. of samples in parentheses)

Dietary group...		Control		Caffeine†	
		µg/mg protein	µg/g fresh wt	µg/mg protein	µg/g fresh wt
Zn	Mean	0.193	26.13	0.149*	21.90*
	SEM	0.012 (4)	0.33 (4)	0.001 (4)	1.47 (4)
Ca	Mean	0.097	13.51	0.064*	9.32*
	SEM	0.007 (4)	1.10 (4)	0.007 (4)	1.18 (4)
Mg	Mean	1.199	160.43	1.004	146.25
	SEM	0.074 (5)	14.00 (5)	0.031 (5)	6.84 (4)

\* Mean values were significantly different from those for the controls ( $P < 0.05$ ).

† For details of procedures, see p. 562.

intravenous injection. A 0.2 ml caffeine solution (1 mg dissolved in physiological saline (9 g sodium chloride/l)) was injected into the femoral vein in the experimental group, whereas 0.2 ml saline was injected into the controls. After 45 min, the animals were killed and their hearts were removed. Ventricular muscle was trimmed of atria, great vesicles and gross epicardial fat and was homogenized for 30 s in 4 vol. 10 mM-sodium bicarbonate (pH 7.0) using a polytron homogenizer at a setting of half-maximal speed. Sarcoplasmic reticulum (SR) vesicles were prepared by the method of Jones *et al.* (1979). All procedures were carried out at 4°. The homogenate was centrifuged twice at 14000 g for 20 min. The resulting supernatant fraction was then sedimented at 45000 g for 30 min. The supernatant fraction was collected and the pellet was resuspended in 0.6 M-potassium chloride, 25 mM-imidazole (pH 7.0) to extract contractile proteins, and then resedimented at 45000 g for 30 min to yield SR.

Usually 3–4 ml supernatant fraction were used and evaporated to 1 ml for determination of Zn content. Approximately 5–10 mg protein cellular fractions were used for determination of Zn content using atomic absorption flame emission spectrophotometry.

(Ca<sup>2+</sup> + Mg<sup>2+</sup>)-ATPase (*EC* 3.6.1.38) activity has been demonstrated to be a good enzyme marker of SR in the heart (Jones *et al.* 1979). The SR was incubated (15 min, 37°) in media containing 25 mM-imidazole buffer (pH 6.8), 100 mM-KCl, 5 mM-sodium azide, 5 mM-magnesium chloride, 0.1 mM-calcium chloride and 3 mM-ATP. The reaction was started by adding SR protein (50–70 mg/ml) and stopped with trichloroacetic acid (100 g/l). Inorganic phosphate was assayed by the method of Fiske & Subbarow (1925). Protein concentrations were measured by the method of Lowry *et al.* (1951). Ca-independent and Mg-dependent ATPase activity was measured in the presence of 1 mM-ethyleneglycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) without adding Ca. The difference between activity measured in the presence of Ca and Mg and that measured in the absence of Ca is referred to as (Ca<sup>2+</sup> + Mg<sup>2+</sup>)-ATPase activity. All chemical reagents were of analytical grade. Data were analysed by using a Student's *t* test, with  $P < 0.05$  being considered significant.

## RESULTS

At the time of killing on day 49, Zn and Ca concentrations of the growing hearts in the caffeine-fed group were significantly less than those of the non-caffeine control group (Table 1). On the other hand, there was no difference in Mg content between the groups.

Zn, Ca and Mg concentrations in the fetal hearts showed no significant differences between caffeine and non-caffeine groups (Table 2). Likewise, there was no difference

Table 2. Zinc, calcium and magnesium concentrations of fetal rat hearts at day 22 of gestation. Caffeine (20 mg/kg body-weight) was added to the 200 g protein/kg maternal diet from day 3 of gestation until day 22 of gestation

(Mean values with their standard errors; no of samples in parentheses. Each determination was the result of values obtained from four pooled hearts from the same litter; however, each sample measurement was from a pooled heart from a different litter)

Dietary group...		Control		Caffeine	
		$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{g}$ fresh wt	$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{g}$ fresh wt
Zn	Mean	0.235	22.82	0.256	23.88
	SEM	0.038 (8)	3.47 (8)	0.013 (4)	1.78 (4)
Ca	Mean	0.451	43.07	0.489	43.80
	SEM	0.068 (6)	6.19 (6)	0.148 (4)	12.47 (4)
Mg	Mean	1.981	187.88	2.106	189.84
	SEM	0.247 (6)	22.30 (6)	0.117 (5)	10.67 (5)

Table 3. Zinc, calcium and magnesium concentrations in hearts of dams. Caffeine (20 mg/kg body-weight) was added to the 200 g protein/kg diet from day 3 of gestation to day 22 of gestation

(Mean values with their standard errors; no. of samples in parentheses)

Dietary group...		Control		Caffeine	
		$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{g}$ fresh wt	$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{g}$ fresh wt
Zn	Mean	0.131	21.44	0.137	22.44
	SEM	0.004 (3)	0.45 (3)	0.005 (5)	0.64 (5)
Ca	Mean	0.072	11.83	0.092	17.20
	SEM	0.019 (3)	3.47 (3)	0.026 (3)	5.43 (3)
Mg	Mean	0.966	158.53	1.087	171.28
	SEM	0.111 (3)	19.90 (3)	0.069 (5)	7.93 (5)

between the groups in Zn, Ca and Mg contents in the hearts of dams (Table 3). The levels of Zn, Ca and Mg in the fetal hearts were much higher than those of the hearts of dams and 49-d-old rats if the results were expressed per mg protein. On the other hand, the levels of Ca/g wet weight in the fetal hearts were higher than those of the hearts of dams and 49-d-old rats.

The Zn content of total homogenates in the adult heart showed a decrease in the caffeine-injected group compared with the saline-injected control group (Table 4). The decrease in Zn in the caffeine-injected group was found in the supernatant fraction.

The mean distribution (percentage) of the Zn showed that 30% of the Zn content of the homogenate was in the supernatant fraction and 67% was in the pellets (14000 g), which contain cell debris, nucleus and mitochondria. The SR fraction contained only 1% of the total amount of Zn in the homogenate and  $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity (SR-marker enzyme) was 4.6 times higher than that of the homogenate (Table 4). The yield of SR was approximately 0.5 mg protein/g heart.

#### DISCUSSION

In spite of the ubiquity of caffeine in our daily life, chronic caffeine ingestion during the growth period has received little attention experimentally. Furthermore, the manner in

Table 4. *Distribution of zinc in subcellular fractions in adult rat hearts*

(Mean values with their standard errors; no. of samples in parentheses. 0.2 ml physiological saline (9 g sodium chloride/l) or 0.2 ml saline containing 1 mg caffeine was injected into the femoral vein. Animals were killed 45 min later)

Fraction	Zn (%)†	Dietary group			
		Control		Caffeine	
		$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{g}$ fresh wt	$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{g}$ fresh wt
Homogenate	100	Mean 0.138 SEM 0.005 (4)	21.50 0.76 (4)	0.119* 0.005 (5)	18.53* 0.89 (5)
Pellet 14000 g (nuclei and mitochondria)	67	Mean 0.125 SEM 0.005 (3)	14.43 0.61 (3)	0.121 0.001 (3)	13.91 0.11 (3)
Sarcoplasmic reticulum vesicles‡	1.0	Mean 0.184 SEM 0.010 (7)	0.211 0.031 (7)	0.151 0.012 (7)	0.183 0.012 (7)
Supernatant fraction (after 45000 g)	30	Mean 0.162 SEM 0.011 (6)	6.43 0.44 (6)	0.123* 0.006 (6)	4.66* 0.33 (6)

\* Mean values were significantly different from those for the controls ( $P < 0.05$ ).

† Mean distribution of Zn expressed as a percentage.

‡  $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity, which is a sarcoplasmic reticulum marker enzyme, was  $13.9 \mu\text{mol}/\text{mg}$  protein per h in the sarcoplasmic reticulum fraction and  $3.0 \mu\text{mol}/\text{mg}$  protein per h in the crude homogenate.

which continuous intake of caffeine during this period may affect the cardiovascular system in the growing rat is not known.

Zn is reported to play an important role in the maintenance of membrane structure and function (Bettger & O'Dell, 1981), and to alter the permeability of major ions, contractile force and transmembrane potential in the muscle cells (Muneoka *et al.* 1979). Since Zn is needed in wound healing, and atherosclerosis is believed to begin with mini-trauma in the intima of vessels, a low Zn concentration may result in inadequate repair (Vuori *et al.* 1985). It has been shown that Zn levels of the necrotic area in men who died of myocardial infarction are low (Speich, 1982). Chronic caffeine feeding has been reported to result in moderate to severe myocardial fibrosis in growing rats (Johansson, 1981). If this is so, then the structural composition of the heart could possibly have been altered by chronic caffeine ingestion during the growth period.

The amount of heart DNA, as an indication of the cell number, continuously increases post-natally in the rat up to about 60 d before coming to a plateau (Winick & Noble, 1965). Thus, caffeine feeding during this period may have permanent effects, shown by the decreased tissue Zn and Ca concentrations in the growing heart in the present study. This may be at least partially related to the altered cardiovascular performance previously shown (Temples *et al.* 1985, 1987). In contrast to Zn, the effect of caffeine on the mechanism of Ca release of the heart has been extensively studied (Rubtsov & Murphy, 1988; Callewaert *et al.* 1989; Rousseau & Meissner, 1989). These studies suggest that caffeine acts on the SR (storage of Ca) of the Ca-release channel.

Caffeine and its metabolites readily cross the placenta and distribute evenly in fetal tissue (Jiritano *et al.* 1985). Therefore, the present observations of no change in Zn levels in growing fetal hearts in the caffeine group was unexpected. It is possible that growing fetal hearts in utero may be especially resistant to the influence of the amounts of caffeine which passed through the placenta. These amounts could be too small to have an effect on fetal hearts. The prenatal period is the most critical to the growth and development of many organs (Zeman, 1970) and the protective role of the placenta has also been well documented (Nowak & Munro, 1977).

The Zn content of the hearts of dams showed no difference between the caffeine and non-caffeine groups. In contrast, the reduced Zn levels in the hearts of 49-d-old rats given caffeine could be recovered relatively quickly, through the body's reserves of Zn, when given ample time.

We observed a high concentration of Zn per mg protein in fetal hearts relative to those of the dams or 49-d-old rats. This is related to the large amount of water in fetal tissue.

Divalent cations such as Ca and Zn are bound to protein and play a variety of important roles in the cells. One of the functions of Ca and Zn is to protect cells against certain kinds of membrane damage (Pasternak, 1988).

In the study of the subcellular distribution of Zn the content of Zn in SR was only 1% of the total amount of Zn in the homogenate, similar to that found in skeletal muscle (Cassens *et al.* 1967). Purification of SR in the present study was verified by the determination of  $(Ca^{2+} + Mg^{2+})$ -ATPase activity (Kithas *et al.* 1989; Quist *et al.* 1989).

Measurement of distribution showed that 30% of Zn content was in the supernatant fraction. Caffeine affected the level of Zn only in this fraction. Zn present in the soluble fraction is protein bound (Smeyers-Verbeke *et al.* 1977). Zn induces the synthesis of and binding to metallothionein (MT), a low-molecular weight cytoplasmic (supernatant) protein. Zn bound to MT is released after degradation of the protein in lysosomes (Cousins, 1985; Bremner, 1987*a*). There is also some evidence to suggest that MT is secreted intact along with its metals from the cell (Bremner, 1987*b*). MT is involved in detoxification (Webb, 1972; Hamer, 1986), storage of Zn (Cousins, 1985; Dunn *et al.* 1987), absorption (Richards & Cousins, 1977) and Zn metabolism (Richards, 1989), and MT formation and degradation respond readily to fluctuations in dietary Zn level (Richard & Cousins, 1975; Fleet *et al.* 1988).

Currently the role(s) of caffeine in the formation, degradation or release of this protein on growing hearts is not known. The effects of caffeine on regulatory functions in MT relative to Zn metabolism in hearts may provide some clarification to understand the present observations.

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