Fluoride Interactions: From Molecules to Disease

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Abstract: Fluoride has long been known to influence the activity of various enzymes *in vitro*. Later it has been demonstrated that many effects primarily attributed to fluoride are caused by synergistic action of fluoride plus aluminum. Aluminofluoride complexes have been widely used as analogues of phosphate groups to study phosphoryl transfer reactions and heterotrimeric G proteins involvement. A number of reports on their use have appeared, with far-reaching consequences for our understanding of fundamental biological processes. Fluoride plus aluminum send false messages, which are amplified by processes of signal transduction. Many investigations of the long-term administration of fluoride to laboratory animals have demonstrated that fluoride and aluminofluoride complexes can elicit impairment of homeostasis, growth, development, cognition, and behavior. Ameliorative effects of calcium, vitamins C, D, and E have been reported. Numerous epidemiological, ecological, and clinical studies have shown the effects of fluoride on humans. Millions of people live in endemic fluorosis areas. A review of fluoride interactions from molecules to disease is necessary for a sound scientific assessment of health risks, which may be linked to the chronic intake of small doses of fluoride and aluminum from environmental and artificial sources.

Key Words: Fluoride, aluminofluoride complexes, G proteins, animal modeling, human exposure, neurotoxicity.

INTRODUCTION

The use of fluoride in laboratory investigations helped in the discovery of glycolytic and Krebs-cycle pathways. The finding by Rall and Sutherland in 1958 that adenylyl cyclase (AC) is activated by fluoride started the era of new discoveries on signal transduction processess [1-4]. During the last two decades, there are numerous references of laboratory studies involving fluoride alone or in combination with aluminum ions (Al^{3+}), mostly investigating its role as a general pharmacological activator of G protein-coupled systems. The effects of fluoride have been studied in many cells/tissues *in vitro* and whole organisms *in vivo*. The expanding research provides evidence that fluoride affects life processes from fertilization to ageing, from gene transcription to cognition with powerful efficacy [5, 6]. In addition to the interpretation of laboratory investigations using isolated cells/tissues or animal models, many epidemiological, ecological, and clinical studies have shown the effects of fluoride on domestic animals and humans.

Dean´s reports formed the foundation of the concept that the ingestion of fluoride will harden the surface of teeth and make them less susceptible to dental caries [7, 8]. The artificial fluoridation of drinking water as a way of preventing dental caries has been in practice for over 50 years in several countries. The past 50 years have seen a dramatic increase in the volume of man-made industrial fluoride compounds released into the environment. Fluoridation of drinking water as well as the use of aluminum sulfate as a flocculating agent in water treatment plants, in addition to the wide use of fluoride and Al^{3+} in medicine, industry, and agriculture, started the era of supplementation of living environment with these ions as never before in the history of human race [9, 10]. Dental fluorosis as the sign of fluoride overload is endemic in at least 25 countries across the globe. Millions of people live in endemic fluorosis area. WHO recently estimated that 2.7 million people have skeletal fluorosis in China, over 6 millions suffer this crippling bone disease in India. Carlsson concerns about what increased fluoride levels would do to the developing brain of newborn infants [11] have gained renewed significance in light of recent findings concerning fluoride and Al^{3+} potential neurotoxicity.

The objective of our article is to provide a comprehensive review of fluoride and aluminofluoride complexes interactions with some components and processes of signal transduction. Such knowledge could help to increase scientific understanding of health risks linked to the chronic but cumulative intake of small doses of fluoride plus Al^{3+} from environmental and artificial sources.

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MECHANISMS OF FLUORIDE ACTION

The highly electronegative fluoride ion with the same size and the same valence orbital as oxygen became the useful laboratory tool in our understanding of the biochemical and biophysical mechanisms of enzyme catalysis underlying biological processes as metabolism and signal transduction. Of particular interest is the ability of fluoride to induce free radical generation and lipid peroxidation in the brain.

1. The Effects of Fluoride on Metabolic Enzymes and Energy Metabolism

The most important enzyme of carbohydrate metabolism inhibited by fluoride is enolase, which changes 2-phosphoglycerate to phosphoenolpyruvate and is intimately related to anaerobic production of energy in glycolysis [12]. The inhibitory effect of fluoride on enolase activity was later identified to be through competition with magnesium (Mg^{2+}) [13]. The competition with Mg^{2+} seems to be also a mechanism of fluoride inhibition of the group of inorganic pyrophosphatases, which catalyze one of the oldest and most common reactions in cells [14, 15]. Fluoride interacts first with the Mg^{2+} on the enzyme in a readily reversible reaction causing a 90% decrease of the catalytic activity. Thereafter, a slow isomerization of the enzyme substrate complex takes place, resulting in a complete loss of activity [16].

Lunardi *et al*. [17] reported that the inhibition of mitochondrial F-ATPase by fluoride requires the presence of Al^{3+}. Prior incubation with the Al^{3+} chelator deferoxamine markedly slowed inactivation, whereas adding 1 μM $AlCl_3$ speeded it. Missianen *et al*. [18] studied the fluoride effect on the Ca^{2+}-Mg^{2+}-ATPase of the endoplasmic reticulum and provided evidence that the time course of inhibition and the concentrations of fluoride and Al^{+} required for this inhibition differ for enzymes from different tissues. The mechanism of fluoride inhibition of P-type cation-transport ATPases has been suggested by the action of aluminofluoride complexes (AlFx), which act as phosphate analogues [17-19].

The experimental evidence indicates that the effects of fluoride on some metabolic enzymes might be attributed to the action of fluoride alone. For example, fluoride has been often used as the inhibitor of various tyrosine and serine/threonine protein phosphatases. Nevertheless, in many cases the biological activity of fluoride is realized by synergistic action of fluoride plus Al^{3+} [20-22]. Ta-

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bles **1** and **2** summarize the observed effects of fluoride on various enzymes with indication of the requirements for Al^{3+}.

2. AlFx Intervention into Phosphoryl-Transfer Reactions

The average stoichiometry of AlFx depends on the fluoride concentration and the pH of the solution [14, 84, 85]. For most of the physiological and biochemical studies involving the putative AlFx, the fluoride source is usually NaF and the Al^{3+} source is $AlCl_3$. Moreover, Al^{3+} is a frequent contaminant of commercial chemicals and it can be picked up from the glass surface, depending on the substance stored in the glass container [86]. The phenomenological observations seemed to verify that pH determines the complexation state of AlFx [20, 87]. The theoretical calculation of the Al^{3+}-fluoride predominance is demonstrated on Fig. (**1**). However, the exact structure and the proportions of species such as AlF_3 and tetrafluoroaluminate anion (AlF_4^{1-}) able to simulate PO_4^{3-} group in many biochemical reactions are still disputed [22, 88]. Al^{3+} forms stronger complexes with fluoride than with the other halides. Out of 60 metal species, Al^{3+} is surpassed only by Sc^{3+} in forming the stronger bond to fluorine [89]. AlF_3 is the most thermodynamically stable compound of fluorine and Al^{3+}.

The ability of these complexes to simulate phosphate groups in many biochemical reactions has been documented by numerous studies. AlFx can bind to proteins by hydrogen bonds to the fluorine atom just as with oxygen atoms of a phosphate ion. Analogies between phosphate group and AlFx consist in atomic and molecular similarities. The fluorine atom has the same size and the same valence orbital as oxygen. Aluminum is close to phosphorus; their valence electrons are in the same shell. An Al-F bond is the same length as a P-O bond in phosphate, i.e.1.5 to 1.6 Å. Like phosphorus, aluminum has possible coordination numbers of 1 - 6, due to the possible hybridization of its outer shell 3p electrons with the 3d orbital. However, an important functional difference between a phosphate group and the structurally analogous AlFx exists [20]. In phosphate, oxygen is covalently bound to the phosphorus and does not exchange with oxygen from solvent, while in the AlFx the bonding between the electropositive Al^{3+} and the highly electronegative fluorine is more ionic in character, allowing fluorine in the bound complex to exchange freely with fluoride ions in solution. While the reaction of a bound phosphate with orthophosphate is endergonic and slow, the corresponding reaction with AlFx is rapid and spontaneous.

Table 1. Inhibitory Effects of Fluoride on Enzymatic Activities. Al Dependency: Yes Means that Al^{3+} is Required, not Required (NR) Means that it has been Examined, not studied (NS) Means that the Presence of Al^{3+} Contamination is not Excluded

ENZYME	SOURCE	NaF	Al^{3+}	REFERENCES
acid phosphatase	ram semen osteoblasts osteoclasts bone marrow, kidney	20-200 µM mM mM <0.5 mM	NS NR Yes NS	[23] [24] [25] [26, 27]
aconitase	liver	mM	NS	[28]
adenylyl cyclase	liver fibroblasts	up to 10 mM 5 mM	Yes Yes	[29] [30]
AChE	red blood cell brain	0.01-10 mM 5-50 mM	Yes NS	[6] [31]
arginase	liver, kidney	>4 mM	NS	[32]
BuChe	blood plasma	50 µM	NS	[33]
enolase	red blood cell hepatocytes embryonic cells oral bacteria	1-50mM 3 mM 1mM, 50µM 16-54 µM	NR NR NS NS	[13, 34] [35] [36] [37]
F-ATPase	mitochondria	mM	Yes	[17]
glucose-6-phosphatase	liver	µM	Yes	[38]
glycogen synthase	hepatocytes	2-15 mM	Yes	[39]
IMPase	fibroblasts brain	mM 20 mM	Yes NR	[40] [41]
lactate dehydrogenase	ram semen fetal osteoblast bone marrow	20-200 µM 6 – 60 µM <0.5 mM	NS NS NS	[23] [42] [26]
lipase	pankreas, liver	10 mM	NS	[12]
L-Ca^{2+}channels	heart	10 mM	NR	[43]
Na^+/K^+ ATPase	plasma membrane kidney	1-10 mM 1-10 mM 5mM	NR Yes NS	[44] [18] [45]
PKC	retina	mM	Yes	[46]
PLD	liver, brain, lymphocyte	mM	Yes	[47]
protein phosphatase	liver bone	10 – 50 mM µM	NR NR	[48] [49]
pyrophosphatase	yeast	5 mM	NR	[16]
pyruvate kinase	red blood cell	10-50 mM	NS	[50]
succinate dehydrogenase	heart, liver, kidney	mM	NS	[51, 52]
urease	animal	mM	NS	[12]

Table 2. Stimulatory Effects of Fluoride on Enzymatic Activities. Al Dependency: Yes Means that Al^{3+} is Required, not required (NR) Means that it has been Examined, not Studied (NS) Means that the Presence of Al^{3+} Contamination is not Excluded

ENZYME	SOURCE	NaF	Al^{3+}	REFERENCES
adenylyl cyclase	heart, liver, brain lymphoma cell smooth muscle heart turkey RBC brain kidney	10 mM 10 mM 10 mM 1-10 mM 10 mM 10 mM 10 mM	NS Yes Yes Yes Yes Yes Yes	[2] [53] [48] [54, 55] [56] [57] [58]
alkaline phosphatase	bone cells	10-100 μM	Yes	[59, 60]
aspartate transaminase	ram semen	20-200 μM NaF	NS	[23]
Ca^{2+}-ATPase	heart, muscle SR	1-10 mM	NR	[61]
c PIPsynthase	liver	10mM	NS	[62]
cytidylate cyclase	rat brain	mM	Yes	[63]
ERK	bone	1 – 10 mM	Yes	[64]
glu S-transferase	ram semen	20-200 μM	NS	[23]
$K^+[ACh]_M$ channel	heart	>1 mM	Yes	[65]
K^+ATP channel	heart	mM	Yes	[66]
lactate dehydrogenase	hepatocytes ram semen	1-30 mM 20-200 μM NaF	NS NS	[67] [23]
L-type Ca^{2+} channel	rabbit femoral artery	10 mM	NR	[48]
MAP kinases	lung	5-7.5 mM	Yes	[68]
glycogen phosphorylase	hepatocytes	1-50 mM	augments	[29, 69]
PI 3-kinase	human HepG2 cells and HeLa cells	30 mM	Yes	[70]
PKC	lung macrophages	5-7.5 mM mM	Yes NR	[68] [71]
PLA2	platelets macrophages endothelial cells	5 - 10mM 5 – 10 mM 5 – 20 mM	Yes Yes NR Yes	[72] [73] [71] [74]
PLC	hepatocytes RBC rabbit femoral artery astrocytes	1-50 mM 1 mM 10 mM AlFx intracellulary	Yes Yes Yes Yes	[56, 75] [76] [48] [77]
PLD	platelets lymphocytes rat atria canine cer. cortex	5-10 mM 10-40 mM 10 mM AlFx only	Yes NS Yes Yes	[78] [79] [80] [81]
tyrosine kinase	osteoblasts	1 – 10 mM 50-200 μM 10 – 100 μM	Yes NS Yes	[82] [83] [59]

3. Fluoride Effects on G Proteins

The finding that adenylyl cyclase (AC) is activated by fluoride made no sense in molecular terms at the time [1, 2]. The breakthrough for explanation of the fluoride effects on G proteins led to the observation that Al^{3+} is a requirement for activation of the regulatory component of AC by fluoride [53]. Of 28 other metal tested, only beryllium could substitute for Al^{3+}.

The liver membranes, multi-receptor fat cell system, and the light-activated rhodopsin system provided the first insight that AC is both inhibited and stimulated by two independent processes involving GTP and fluoride [90-92]. In a detailed study of the light-activated rhodopsin system it was suggested that hydrolysis of GTP is a very rapid process, whereas the rate limiting step is the release of inorganic phosphate from its binding sites on transducin, the G protein responsible for activation of phosphodiesterase (PDE) in rod outer segments. Thus arose the nomenclature now popularly known as G_t, G_s, and G_i classes [93]. Beginning with transducin it emerged that G proteins are constructed of three types of subunits, an α-subunit uniquely capable of binding and degrading GTP and a tightly knit complex of β- and γ-subunits. This discovery, eventually established for all G proteins coupled to receptors [3], opened up a new chapter in signal transduction, which, in recent years, has helped to explain the pleiotropic actions of hormones.

Fluoride Plus Al^{3+}: the Tools in the Discovery of the Role of G Proteins

Gilman and co-workers [3, 53] found that the target of the AC activation by fluoride was a heterotrimeric G protein and suggested that the active stimulatory agent was AlF_3. The demonstration that fluoride activation of transducin correlated with the stoichiometric binding of one Al^{3+} to transducin-GDP [19] also led to the suggestion that it is AlFx, which interacts directly with the β-phosphate of GDP. As the heterotrimeric G proteins are activated when they go from the GDP-bound to the GTP-bound state, Chabre [20] suggested that AlFx mimics the role of the γ-phosphate only if the β-phosphate is present and remains unsubstituted on its oxygen. The effect is more readily seen with G proteins because GDP is always tightly bound in the nucleotide site of the protein.

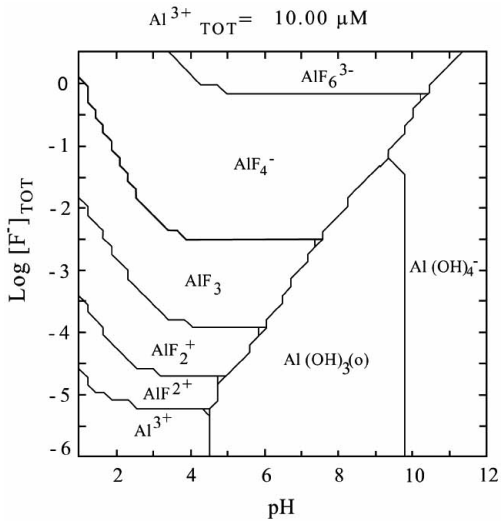

Fig (1). Dependency of the complexation state of AlFx on pH and fluoride concentration for Al^{3+} 10 µM.

Structures of G_t and G_i have been solved in their GTP-, GDP- and AlFx-liganded states [93]. Gα-subunit is composed of essentially two distinct domains, a Ras-like GTPase domain and a predominantly helical domain that is unique to the Gα-subunit. The bound guanine nucleotide is held at the interface of these domains. In the GTP-bound state, the switch regions are held in place by contacts to the terminal γ-phosphate of the nucleotide, whereas these regions appear to be less ordered in crystals of the GDP-liganded G proteins. The determination of the three-dimensional structures of heterotrimeric G proteins bound to GDP and AlFx [94, 95] confirmed that AlFx is located in the γ-phosphate-binding site of these proteins. The studies of the crystal structures of nucleotide binding proteins complexed with fluoride and Al^{3+} indicate that factors other than pH, such as the location of positively charged amino acid of the active site of the phosphoryl-transferring enzyme may cause deviation from the strict pH dependence of AlF_3 versus AlF_4^{1-} in biological systems [22].

AlFx as Pentavalent Transition State Mimic

The assumption that AlFx acts through its tetrahedral phosphate-like complex was supported by the analogy with beryllium because all beryllium complexes are strictly tetrahedral and cannot take on the pentavalent conformation adopted by phosphate in transition states [20]. However, both ATPase and GTPase pathway must go through a pentacoordinated transition state for the γ-phosphate. The later studies of the crystal structures of nucleotide binding proteins brought evidence that AlFx may also act as the phosphoryl transfer transition state analogue with a pentavalent phosphorus [22]. The X-ray crystallography of heterotrimeric G proteins bound to GDP and AlFx [94, 95] brought evidence that AlF_4^{1-} seems to be the active site species. Al^{3+} is bound to four fluoride ligands in a square-planar coordination with two oxygen ligands at the apical position of the resulting octahedron (Fig. **2**). One oxygen ligand is the γ-phosphate oxygen, the leaving group in the transfer reaction, whereas the other is the oxygen from water believed to represent the attacking nucleophile of the hydrolysis reaction. The structure determination both supports the conclusion that AlF_4^{1-} binding mimics the transition state of the reaction. The structures of the two metabolic enzymes nucleoside diphosphate kinase and uridylate monophosphate kinase unexpectedly indicate that AlF_3 is the transition state mimic [87, 96]. The reason for the differences is not clear at present; however, a pentavalent aluminum more closely resembles the real transition state of the phosphoryl transfer reaction.

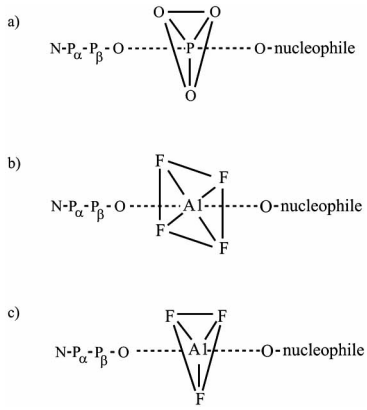

Fig.(2). Conformational changes of the γ-phosphate in a phosphoryl-transfer reaction transition state a) and various species of AlFx: b) AlF_4^{1-}, c) AlF_3. Dotted lines indicate that the degree of bond making and bond breaking determines whether the transition is more dissociative, with a metaphosphate-like intermediate, or associative, with a pentavalent intermediate. Charges have been omitted for clarity. N = guanosine or adenosine.

Fluoride and GTPase Activating Proteins

The GTPase activity of Gα-subunit is regulated by accessory proteins "regulators of G protein signaling" (RGS). The RGS proteins may bind to Gα-subunits accelerating the rate of GTP hydrolysis. The results from a growing number of reconstitution studies indicate that RGS proteins act as GTPase activating proteins (GAPs) of Gα-subunits [93]. The intrinsic GTPase activity of the α-subunit determines the lifetime of the active state of the G protein [4]. Biochemical evidence showed that GAPs bind with higher affinity to G·GDP·AlFx complex of Gα-subunits than to the triphosphate state of G protein, indicating that GAP stabilizes the transition state of the GTPase. This was confirmed by the three-dimensional structure of the GAP·G_{i1}·GDP·AlF_4 complex determined by X-ray crystallography, in which GAP contacts the regions of G_iα-subunit involved in GTP hydrolysis [97].

These findings are complemented and highlighted by the determination of the structure of a complex between RasGAP and Ras·GDP in the presence of Al^{3+} and fluoride ions [98]. The proto-oncogene product Ras is a small G protein that is a component of intracellular signaling pathways involved in cell growth and division. It has a very low intrinsic GTPase reaction rate that is stimulated 105-fold by RasGAPs that downregulate the accumulation of Ras·GTP. Ras binds AlFx only in the presence of RasGAP, and an efficient GTPase site is only created by the addition of stoichiometric amounts of RasGAP [22]. It shows that, in this case AlF_3 forms a pentagonal bipyramid, with the fluorides forming the trigonal base with two apical oxygen ligands. The authors suggest that RasGAP stabilizes the transition state by neutralizing developing charges on the γ-phosphate during phosphoryl transfer.

Subsequently, similar studies demonstrated that several classes of small G proteins can interact with their respective GAPs in the presence of AlFx. Vincent *et al.* [99] reported the ability of fluoride to promote a high-affinity complex between the Ras-related RhoA GTPase and the p190 RhoGAP. Surprisingly, they found that formation of this high affinity complex does not require either Al^{3+} or guanine nucleotide. The possibility of Al^{3+} contamination was examined by the addition of the chelating agents, EGTA and deferoxamine, which can bind Al^{3+}. The chelators did not detectably affect the observed NaF-dependent RhoA-p190 complex. These authors therefore suggested a distinct mechanism of transition state stabilization of small G proteins by fluoride that is not consistent with the phosphate analogue model. However, AlFx can stabilize complexes formed between Ras and RhoA and their corresponding

GAPs [100]. AlFx can convert a small G protein Arf1- GDPcomplex into an active conformation *in vitro* and *in vivo.*

4. The Excitotoxic Process

High fluoride levels cause accumulation of large amounts of free radicals and peroxides by inhibiting superoxide dismutase and glutathione peroxidase activities [101]. It mainly causes denaturation of proteins and peroxidation of membrane lipids with increased permeability of cell membrane. Fluoride and AlFx are both known to generate reactive oxygen and nitrogen species (ROS and RNS) and lipid peroxidation (LPO) products [102-105], which have been shown to enhance excitotoxic damage.

There is compelling evidence that excitotoxicity plays a major, if not central, role in a number of neurodegenerative diseases as well as environmental toxicities. Glutamate and aspartate constitute the major excitatory neurotransmitters in the central nervous system (CNS). At low concentrations for brief periods of time, glutamate acts as a neurotransmitter and can even have neurotrophic effects. Yet, higher concentrations over a longer period of time are associated with a series of reactions that can lead to synaptic disruption, dendritic retraction or cell death via necrosis and/or apoptosis. Synaptic loss and dentritic retraction are the earliest reactions and can occur at doses of glutamate below those needed to produce neuron death. Intense, or prolonged interactions of glutamate, or other excitatory molecules, with the glutamate receptors can initiate a series of other destructive reactions that include ROS and/or RNS (especially peroxynitrite), LPO products, the proinflammatory ecosanoid pathways, immune hyperactivity and depressed mitochondrial energy production.

Glutamate receptors are divided into ionotropic (ion-gated) and metabotropic receptors. The inotropic receptors consist of NMDA, AMPA and kainate receptors, each with a specific gating mechanism, distribution and physiological activity. Yet, all three are modulated through direct phosphorylation by both serine/threonine and tyrosine kinases. These ionic receptors have been further characterized by a number of subunits through cloning techniques to include specific patterns of these subunits. For example, NMDA is composed of NR1 and one or more of the NR2A-D subunits. AMPA is composed of GluR1-4 and kainate is encoded by GluR5-7 subunits. These subunits greatly increase the specificity and diversity of reactions possible. In addition to the ionic receptors there are three classes of metabotropic glutamate receptors with eight subtypes thus far identified. These receptors are seven-α-helix receptors connected to G proteins. In the excitatory synapse the metabotropic receptors are arranged along the periphery of the postsynaptic density, with the ionic receptors within the core [106]. Gegelashvili and co-workers [107] demonstrated that metabotropic receptors play a modulatory role on the glutamate transport system. The AlFx complex can activate these receptors since they are operated by G protein systems.

There is an intimate interaction between the metabotropic receptors and NMDA and AMPA receptors, allowing rapid modulation of the excitatory unit. Recent studies indicate that group I and II metabotropic receptors can enhance excitotoxicity through their regulatory effects on the NMDA receptor [108]. Within the postsynaptic density there also exist actin, tubulin, scaffolding proteins and an array of regulatory molecules such as protein kinase A (PKA), protein kinase C (PKC) and protein phosphatase-1. These help direct the information intracellularly and amplify and sharpen the signal.

Because glutamate is excitotoxic when existing in the extracellular space, a glutamate gradient ratio of intracellular to extracellular concentration of 1000 to 1 exist in nervous tissue. This gradient is maintained by a sodium-coupled, high affinity transport system on both the membranes of nerve terminals and fine astrocytic processes. Cloning techniques have identified five glutamate transporters [109]. The interactions between various types and subtypes of glutamate receptors, second messenger molecules, eicosanoid metabolites, ROS, RNS, and phosphorylating enzymes makes control of these protective systems extremely complex. Brain protection by the glutamate transport system has been shown to be vulnerable to a number of toxicities, including free radicals, cytokines, arachidonic acid (AA), proinflammatory eicosanoids and Al^{3+} [110]. When overactivated, glutamate ionic receptors trigger a series of intracellular events that include PKC activation with phosphorylation of PLA_2, which in turn catalyzes the release of AA from the plasma membrane [111]. This triggers the proinflammatory eicosanoid pathways, leading to microglial activation, inflammation, cytokine release and oxidative stress.

By opening the Ca^{2+} pore through activation of the NMDA receptor, the rising cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) activates inducible nitric oxide (NO) synthetase, leading to accumulating concentrations of NO. In conjunction with the elevation of superoxide generated by eicosanoid activation and other radical activators, we see a reaction between superoxide and NO that produces the very destructive peroxynitrite radical. Peroxynitrite has been shown to damage mitochondrial energy-producing enzymes, mDNA and mitochondrial membranes, leading to low cellular energy levels [111]. With severe damage to the mitochondria, cytochrome c is released into the cytosol, which activates caspase 3, leading to apoptosis. Peroxynitrite, along with the hydroxyl radical, have also been shown to inhibit the glutamate transport proteins, thereby further increasing extracellular levels of glutamate [110, 112]. This viscous cycle leads to increasing synaptic and dendritic damage and eventual neuronal death. Toxicity of Al^{3+}, fluoride and AlFx have all been shown to be connected to these various processes. Further interactions of fluoride and AlFx have recently been reviewed [101].

THE EFFECTS OF FLUORIDE AND ALFx ON VARIOUS CELLS/TISSUES *IN VITRO*

The use of fluoride in medicine opened the need to study the effect of fluoride on various biological processes. The effects of fluoride *in vitro* have been thus studied in most of the cells/tissues of animal or human organisms. Fluoride activation in the presence of trace amount of Al^{3+} has been often used as evidence for involvement of a heterotrimeric G protein in a system [5, 22]. Many of these studies also provide evidence that fluoride influences various functions and biochemical reactions of many cells and tissues of the animal kingdom. On the other hand they provide evidence about the pharmacological efficacy of fluoride and a small molecule, which is AlFx.

1. Photoreceptor Cells

The retinal transducin cascade represents the most accessible G protein system to study. The cGMP PDE plays a central role in visual excitation in vertebrate rod photoreceptor cells. Absorption of a photon by the photopigment rhodopsin leads to activation of the PDE which, in turn, catalyzes the hydrolysis of cGMP, causing closure of cGMP-gated cation channels and hyperpolarization of the cell membrane [113]. Bigay *et al.* [19] demonstrated that AlFx activate stoichiometric amounts of transducin in the micromolar range. This activation requires the presence of GDP in the nucleotide site of transducin α-subunit. Purified α-subunit activates purified cGMP PDE in the absence of photoactivated rhodopsin and in the presence of fluoride plus Al^{3+}. It has been reported that transducin serves as a high affinity substrate for PKC in its native form in intact rod membranes. Sagi *et al.* [46] reported that AlFx inhibited PKC mediated phosphorylation of purified transducin α-subunit.

2. Hepatocytes and Liver

Hepatocytes maintain responsiveness to hormones and serve as model cells equipped with very complex biochemical pathways.

The stimulation of glycolysis by vasopressin, angiotensin II, and α_1-adrenergic agonists is mediated in the liver through the increase of the $[Ca^{2+}]_i$ [29, 69, 75]. It has been demonstrated that the phosphoinositide (PPI) signaling pathway [114, 115] is activated and involved in these events.

Blackmore with coworkers [39, 75] demonstrated in their studies that the treatment of isolated hepatocytes with NaF produced the efflux of Ca^{2+}, rise in $[Ca^{2+}]_i$, the decrease in phosphatidylinositol 4,5-bisphosphate (PIP_2) content, the increase in inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (DAG). The lowering of cAMP induced by AlFx was mediated by the G_i. $AlCl_3$ potentiated the effects of low doses of NaF (2-15 mM) and deferoxamine abolished this potentiation. Fluoride in the presence of Al^{3+} thus mimicked the action of Ca^{2+}-mobilizing hormones glucagon and vasopressin in hepatocytes. Vasopressin-stimulated $Ins(1,4,5)P_3$ formation was evident in the presence of GTP or GTP(S) in a purified rat liver plasma membrane preparation. The effects of submaximal doses of AlFx were potentiated by submaximal doses of glucagon, vasopressin, angiotensin II, and α_1-adrenergic agonists. The conclusion was made that AlFx mimics the effects of Ca^{2+} mobilizing hormones by activating the G protein, which couples the hormone receptor to PLC.

Fluoride has been used as the laboratory tool for the study of prostaglandylinositol cyclic phosphate (cPIP), which is endogenous cAMP antagonist [62]. NaF activates the plasma membrane-bound enzyme cPIP synthase, which combines prostaglandin (PG) E and inositol phosphate to cPIP. Preincubation of liver plasma membranes with the tyrosine kinase src kinase causes a 2-fold increase of cPIP synthase activity. The authors conclude that inactivation of the enzyme is connected with protein tyrosine defosforylation. The cPIP degrading activities have been found in all rat tissues tested, but are highest in the liver and lowest in the brain [116]. Inhibition of PG synthesis by the drug indomethacin suppresses the synthesis of cPIP in rat liver and leads to a metabolic state comparable to diabetes type 2 [117]. These authors also observed the stimulation of cAMP synthesis by fluoride in indomethacin-treated rats. Fluoride in the presence of Al^{3+} thus affects the liver as an organ involved in glycogenolysis, fatty acid oxidation, and lipolysis.

3. Cells of Blood and Immune System

The investigators in the first three decades of the last century tried to solve the simple question whether fluoride stimulates clotting of blood, attributing fluoride effect to a retardation of glycolysis [12]. Multiple external signals and signaling systems that are involved in the function of various blood elements were described during the past three decades [118].

Platelets

The majority of platelet physiological agonists stimulate the breakdown of PIP_2, the generation of $Ins(1,4,5)P_3$ plus DAG, and mobilization of Ca^{2+} from the dense tubular system [119]. Following the increased $[Ca^{2+}]_i$ induced by $Ins(1,4,5)P_3$, the Ca^{2+}-dependent PLA_2 releases AA from membrane phospholipids and thromboxane (TX) synthesis is activated. Activation of platelets is connected with shape changes. Rendu *et al*. [78] observed that incubation of platelets with NaF (5-10 mM) induced only slight morphological changes. Addition of 10 μM $AlCl_3$ resulted in aggregation. One minute after addition of $AlCl_3$, most of the granules were concentrated in the center of the cell, but some were extruding their contents by direct exocytosis. According to authors, this observation suggests that the active species for platelet activation was AlF_4^{1-}.

Padfield *et al*. [120] examined the effect of AlFx on G protein control secretion from α- and dense-core granules in human platelets. As shown for permeabilized platelets, Ca^{2+} alone stimulated a concentration-dependent increase in 5-hydroxytryptamine (dense-core-granule marker) and platelet-derived growth factor (α-granule marker) release. Neither GTP(S) nor AlFx appeared to have a significant effect on Ca^{2+}-dependent release from α- and dense-core granules. GTP(S) stimulated Ca^{2+}-independent release from both α- and dense-core granules. In contrast, AlFx had no effect on Ca^{2+}-independent release from either α- or dense-core granules. Padfield with co-workers based their interpretation on the presumption that GTP(S) can activate both heterotrimeric and small G proteins in platelets, whereas AlFx activates only heterotrimeric G proteins and suggested that the secretion is regulated by a small G protein in the human platelets.

Red Blood Cells (RBC)

RBC provided the model for the discovery of fluoride inhibition of enolase *in vitro*. A significant reduction in the content of ATP and ADP and an increase in the content of AMP in RBC was also found in rats after 4 weeks of exposure to 4 or 16 ppm NaF [121].

Avian RBC have contributed enormously to our understanding of β-adrenergic regulation of AC via the G_s protein and understanding the mode of activation of PLC by a G protein [56, 122]. $G_{q/11}\alpha$-subunit has been purified, sequenced and cloned from turkey RBC [123]. This protein has the capacity to activate PLC in an AlFx-dependent manner. Reconstitution of [^{3}H]inositol-labeled turkey RBC membranes with G protein β/γ-subunits resulted in inhibition of AlFx-stimulated PLC activity and in AlFx-stimulated AC activity [124].

Plasma membrane of human RBC is not equipped with receptor molecules. The question of why human RBC maintain a high turnover rate of PPI remains unanswered. Surprisingly, the presence of G proteins and PLC was reported [118]. However, no physiological agonist evoking PLC activation has been found. Fluoride in the presence of Al^{3+} seems to be one of the rare stimuli, which is able to activate PLC, to induce PPI hydrolysis and, in parallel, to evoke shape changes of human RBC [76]. AlFx increased the level of $Ins(1,4,5)P_3$ and released Ca^{2+} from the RBC plasma membrane. Shape changes and disorganization of the tubulin structure were observed. It has been reported that AlFx may impair the polymerisation-depolymerisation cycle of tubulin coupled to the hydrolysis of bound GTP into GDP [20]. AlFx can bind to tubulin molecules in GDP-β phosphate and thus mimic the GDP+P intermediate state. Their binding affinity is three fold higher than that of phosphate.

Neutrophils

Rapid and dynamic changes of the actin network are of vital importance for the motility of human neutrophils. AlFx, in combination with GDP, stimulates actin assembly in electropermeabilized neutrophils and could be totally abolished by GDP(S) [125, 126]. This effect parallels an increase in $[Ca^{2+}]_i$, indicating that PLC is activated. The binding of ligands to chemoattractant receptors in human neutrophils resulted in a rapid association of these receptors with a cytoskeletal fraction and a specific activation and release of $G_{i2}\alpha$-subunits from this fraction. Sarndahl *et al*. [127] observed that GTP(S) or AlFx not only caused a release of $G_{i2}\alpha$-subunits from the cytoskeleton but also an association of chemoattractant receptors with the cytoskeleton. Adhesion and chemoattractant receptors are known to trigger activation of the small G protein Ras in human neutrophils, but the signaling mechanism that activates Ras has only been partially elucidated [128].

Lymphocytes

Lymphocytes with their central role in immunity attract a great deal of laboratory investigations. The binding of antigen to the multicomponental T cell receptor activates several signal transduction pathways. Activation of PLC represents one of them. T cell receptor activation has been shown to cause an increase in tyrosine phosphorylation [129]. Regulation of the development of thymocytes into mature T cells within the thymus is now known to involve antigen-induced deletion, by apoptosis, of potentially autoreactive thymocytes. Stimulation of immature thymocytes or of mature T

cells through their T cell receptor complex activates PLC. The treatment of thymic lobes cells with fluoride in the presence of Al^{3+} provoked apoptosis of a wider range of thymocyte subtypes and such stimulation also provoked an accumulation of inositol phosphates (InsPs). The responses to AlFx were not prevented by inhibitors of tyrosine kinases, suggesting that unidentified G proteins, which couple to PLC activation may also be capable of initiating apoptosis by a route independent of the T cell receptor. AlFx stimulated PLC activity and PPI turnover was observed in resting T cells of autoimmune-prone mice, mature $L3T4^+$ and Ly^{2+} double-negative T cells from normal thymus, and from enlarged lymph nodes of autoimmune-prone mice [130]. Increased $[Ca^{2+}]_i$ induced by AlFx has been observed in cloned helper T lymphocytes.

AlFx mimicked CD2-, CD3-, and CD43- mediated Ca^{2+} responses in T lymphocytes derived from human peripheral blood and in leukemic T cell line [131, 132]. Later studies revealed that NaF augments the human lymphocyte response from human blood to a mitogen (phytohemagglutinin, PHA) or a specific morbilli antigen [133]. The cytokine interferon-γ released from activated human T lymphocytes and/or NK cells, was significantly increased when whole blood cells were simultaneously incubated with fluoride. The authors suggest that the ability to influence interferon-γ release during an immune response could be one of the primary means by which the fluoride ion influences the immune system.

AlFx enhanced eicosanoid synthesis in macrophages [73]. NaF led to *in situ* activation of PLC, PKC, and PLA_2. NaF was shown to reduce intracellular ATP levels, to suppress agonist-induced protein tyrosine phosphorylation, and ROS formation. Addition of $AlCl_3$ or deferoxamine had little or no effect on NaF-mediated enzyme activation. Goldman *et al*. [71] therefore suggested that at least some of the pleiotropic effects of fluoride in intact macrophages might not be mediated by G protein activation but rather by depletion of ATP.

4. Heart

Fluoride and AlFx stimulate AC activity in the heart [2, 54, 55]. Hrbasova *et al*. [55] reported that the stimulation of AC activity was 6 times higher after the addition of 10 mM NaF + 500 μM $AlCl_3$ than the stimulation by GTP and 4.4 times higher than the stimulation by isoproterenol in the right ventricle. It seems, that $G_s\alpha$-subunit is involved in mediating fluoride stimulation of cardiac AC. In rat atria the rate of fluoride-induced PLD activation (and mass production of DAG) was maintained for at least 60 min [80]. Experimental evidence suggests that the myocardial PLD-PA phosphohydrolase-signaling pathway may regulate Ca^{2+} movements and contractile performance of the heart. Williams *et al*. [134] suggested that the increased production of DAG by PA phosphohydrolase might lead to impairment of Ca^{2+} homeostasis associated with cardiomyopathy.

Yatani *et al*. [65] studied a mechanism of fluoride activation of G protein-gated muscarinic atrial K^+ channels. They applied KF to the cytoplasmic face of inside-out membrane patches excised from guinea pig atria. Fluoride activated single K^+[ACh] channel currents in both a concentration- and a Mg^{2+}-dependent manner, while deferoxamine inhibited this activation. At low concentrations of KF (<1 mM), micromolar Al^{3+} potentiated KF stimulation. When ATP closed cardiac ATP-sensitive K^+ channels, activators of endogenous G proteins, GTP, GTP(S), or AlFx stimulated channels [66]. Fluoride (1-75 mM) also increased the activity of the L-type Ca^{2+}channel dose-dependently [135]. Fluoride had no effect on the Ca^{2+} channel activity when the myocytes were pretreated with a potent inhibitor of protein kinases, indicating that fluoride increased the Ca^{2+}channel activity via modulation of the phosphorylation state of the channel protein.

5. Kidney Cells

Fluoride has been considered to be a nephrotoxic substance. The effects of fluoride plus Al^{3+} on the kidney have been studied *in vitro* using glomerular mesangial cells, proximal tubular cells, and the collecting tubule cells of rat kidney. The transepithelial movement of fluids, electrolytes, and larger molecules is achieved by the activity of specialized transporting proteins, including enzymes, receptors, and channels, that are located on either the apical, basal, or lateral plasma membrane domains of kidney epithelial cells [136, 137]. Fluoride and Al^{3+} in kidney tubular cells were found to affect ion transporting processes, stimulate AC, inhibit amiloride-sensitive Na^+/H^+ exchange regulated by cAMP-dependent protein kinase, enhance epidermal growth factor-stimulated PG production, and mimic vasopressin and bradykinin induced Ca^{2+} mobilization. Acid phosphatases were suggested as the potential cellular targets of fluoride action in the renal tissue [27]. A 9.3 fold peak increase in the AC activity was observed in the basolateral membranes incubated with fluoride plus Al^{3+} [58]. Exposure of intact cells of the rat inner medullary collecting tubule to fluoride enhanced both basal and epidermal growth factor-stimulated PG production in the presence of Al^{3+} [138]. After 24 h of exposure, 5mM fluoride decreased cell number (-23%), total protein content (-30%) and increased LDH release (+236%) in human and rabbit collecting duct cells and Henle's loop. The Na^+/K^+ ATPase activity was inhibited (-58%) [45].

6. Lung Endothelial Cells

Lung inflammatory response has been observed as the symptom of fluorine intoxication. Lung endothelial cells (EC) play an important role in the inflammatory process by releasing cytokines in a complex cell to cell network. Interleukins (IL) are important mediators of this cell signaling. Exposure to fluorides can induce inflammatory reactions, cell cycle arrest, and apoptosis in different experimental systems. NaF has been reported to induce a strong IL-8 response in human lung EC via mechanism that seems to involve the activation of G proteins [68]. NaF induced sustained increase in PKC activity. In contrast, the PKC activator TPA induced a relatively strong, but transient effect and augmented the NaF-induced PKC activity. Inhibition of the mitogen-activated protein kinase (MAPK) p38 partially reduced the IL-8 response to NaF. The NaF-induced IL-8 response was weakly augmented by forskolin and the G_i inhibitor pertussis toxin. These data suggest that NaF-induced increase of IL-8 in human lung EC involves PKC- and MAPK p38-linked pathways.

The inhibition of proliferation by NaF in the human lung EC line was observed [139]. NaF induced apoptosis with a maximum at 5-7.5 mM after 20 hours of exposure. The number of cells with plasma membrane damage increased moderately up to 5 mM, but markedly at 7.5 mM. Deferoxamine almost completely prevented the NaF-induced responses, which may suggest a role for G protein activation. NaF induced a weak but sustained increase in PKC activity. Using various pharmacological tools these authors concluded that activation of MAPK p38 and c-jun-NH_2-terminal kinase (JNK) are involved in the NaF-induced apoptosis. The tyrosine kinase inhibitor genistein also markedly reduced the NaF-induced apoptosis, whereas the phosphatidylinositol 3-kinase (PI 3-K) inhibitor wortmannin augmented the response.

7. Brain

Fluoride, AlFx and Al^{3+} are all known to interfere with a number of glycolytic enzymes, including enolase, phosphofructokinase, aconitase, pyruvate dehydrogenase and ATPase. Al^{3+} has been shown to inhibit pyruvate dehydrogenase with a resulting decline in acetyl-CoA. This results in a significant suppression of cellular energy production [140]. NaF has been shown to inhibit glycolytic enzymes *in vitro*, with a loss of mitochondrial membrane potential, caspase 3 and 9 elevation, DNA fragmentation and eventual apoptosis [141]. A number of studies have shown that when neuronal cellular energy production and/or Mg^{2+} are deficient, no matter the cause, excitoxicity is greatly enhanced - so much so that even

physiological levels of glutamate or other excitatory amino acids can produce excitotoxicity [142]. There is compelling evidence that excitotoxicity plays a major, if not central, role in a number of neurodegenerative diseases. It is known that elevation of ROS, RNS, and LPO products act as nonspecific activators of glutamate triggered excitotoxicity in the brain. Likewise, both fluoride and AlFx are known to increase brain oxidative and nitrative stress and LPO. Because of the intimate link between elevations in brain ROS and RNS and LPO products and excitotoxicity, we can be confident that the latter process most likely plays a critical role in fluoride neurotoxicity.

The family of cell-surface receptors that require coupling to G proteins is vast and diverse in the brain [143, 144]. PPI was shown to turn over rapidly in the brain and participate in many processes of neurotransmission. Fluoride salts in the presence of Al^{3+} have been often used to stimulate PPI hydrolysis in laboratory experiments *in vitro*. The ability of AlFx to mimic the effects of Ca^{2+}-mobilizing hormones suggests the coupling of hormone receptors to PPI breakdown through G proteins [145].

Impaired Glutamate Reuptake

Direct toxicity of fluoride or AlFx to the major glutamate transport proteins, GLAST and GLT-1 has not been shown, mainly because no one has looked at the possibility. The brain contains five sodium-coupled, high affinity glutamate transport proteins [146]. It is known that PKC plays a vital role in the availability of glutamate transport molecules [147] as well as those for serotonin [148], GABA [149], norepinephrine [150], and dopamine [151]. Because fluoride is known to stimulate activity of PKC, one would expect elevations in fluoride to improve glutamate clearing by the glutamate transport proteins. Yet, fluoride and AlFx are both associated with significant induction of ROS and LPO products that are known to inhibit glutamate transport proteins as discussed above. In addition, both induce the release of AA from cell membranes, and AA is a rather potent inhibitor of glutamate transporters as well [152]. In addition, PKC is known to play a role in microglial activation and glutamate release [153], as well as a central role in glutamate toxicity [154]. Blocking PKC significantly reduced excitotoxic damage *in vitro*.

It has also been shown that PI 3-K plays a modulatory role in glutamate transporter activity as well. Davis *et al*. [155] demonstrated that PI 3-K inhibitor wortmanin inhibited glutamate uptake in a glioma cell line by 35%, indicating less than complete control. This represents another phosphorylation reaction that could be influenced by AlFx. PI3-K is upstream to PIP_2, which forms Ins(1,4,5)P_3 and DAG, regulators of intracellular Ca^{2+} and PKC activation, respectively. Guillet *et al*. [156] found PKA, PKC and PI 3-K to all to be involved in glutamate uptake. In addition, direct phosphorylation of GLAST-1 has been shown to inhibit its activity [157].

Hypomagnesmia

Zeevalk and Nicklas [158] found that the potency of glutamate excitotoxicity was enhanced two to five-fold in the absence of Mg^{2+} and that it reduce the minimal concentration of agonist needed from 25 μM to 5 μM and 300 μM for NMDA and 300 μM to 10 μM for glutamate. As we have seen, fluoride and AlFx stimulate PKC activity. One study found that activation of PKC enhanced excitotoxicity and appeared to do so, at least in part, by interfering with Mg^{2+} blockade of the NMDA receptor [159]. It has also been shown that Na^+/K^+ ATPases are necessary for maintaining the voltage-dependent Mg^{2+} block of NMDA receptors [160]. Previous studies indicated that AlFx (most likely as AlF_4^{1-}) inhibited the activity of Na^+/K^+ ATPase and that they do so by way of G protein-coupled receptors [161]. Taken together this would strongly suggest that AlFx could interfere with neuronal protection from NMDA receptor overactivity (excitotoxicity), especially in the face of low Mg^{2+} or neuronal energy levels.

Microglial Cells

The microglial cells are the resident immune cells in the CNS. Under resting conditions microglia are quiescent but are easily activated by a number of insults, including trauma, infections, heavy metals, systemic immune activation, ß-amyloid (Aß), rotenone, stress, oxidized LDL-cholesterol, hypoxia/ischemia and hypoglycemia. While transient, mild to moderate activation can be neuroprotective, chronic activation, especially at high levels, is known to trigger neurodegeneration, especially under certain conditions. Astrocytes regulate intersynaptic communication between neighboring synapses and, probably, overall volume transmission in the brain [162]. When activated, microglia down-regulate surface karatan sulfate proteoglycans and assume ameboid characteristics. Microglia contain a number of surface receptors such as IL-8 and glutamate receptors, and when activated can secrete a large number of molecules including various ILs, TNF-α, chemokines, TGF-ß, matrix metalloproteinases, metalloprotease-disintegrin ADAM8 and elastase. Most importantly, when activated microglia secrete considerable amounts of glutamate, which can reach excitotoxic levels.

Fluoride has been used as an enzymatic inhibitor or as the activator of G proteins in laboratory investigations of astrocytes functioning. Astrocytes respond to extracellularly applied ATP, which causes release of Ca^{2+} from an intracellular Ins(1,4,5)P_3-sensitive pools. Increases in PPI hydrolysis and $[Ca^{2+}]_i$ were elicited by intracellular application of GTP(S) and AlFx in astrocytes from the dorsal spinal cord [77, 163]. AlFx also stimulated the tyrosine phosphorylation of a 42 kDa protein (p42) and activation of p42 MAP kinase in primary cultures of mouse embryo astrocytes [164].

Since no one has examined the possibility of direct microglial activation by fluoride or the AlFx, we must examine other ways they could activate microglia. Fluoride and AlFx are both known to generate ROS and RNS and LPO products [104, 105]. ROS and LPO activate microglia, thus linking both AlFx and fluoride to chronic brain inflammation. Al^{3+} itself is known as an immune adjuvant and it is suspected that Al^{3+} within the brain, especially that complexed with amyloid plaque may activate resident microglia through this mechanism [165, 166].

Cytokine Enhancement of Excitotoxicity

While cytokines are known to play a role in neuroprotection, when chronically elevated or existing within the brain in high concentrations they can be quite neurotoxic. A number of studies have shown enhanced excitotoxicity in the face of elevated inflammatory cytokines [167-169]. ILs and TNF-α have been found to be strongly upregulated during diseases such as Alzheimer's disease (AD), stroke or Parkinson's syndrome [170]. There is considerable cross-talk between cytokines, so that IL-ß and TNF-α, when existing in higher concentrations within the brain, activate neurodegenerative processes, especially in the presence of elevated levels of glutamate [171]. IL-1 has also been shown to mediate the effects on protein τ hyperphosphorylation by microglia through p38-MAPK activation [172]. As stated previously, PKC plays an important role in microglia activation. The entry point for this activation appears to be MAPK. In addition, ERK and JNK were also activated. Once activated by Al^{3+}, fluoride or other triggers, microglia secrete large concentrations of IL-1ß and TNF-α, which can then recruit more microglial activation in a vicious cycle that ultimately leads to neurodegeneration. This is suspected to be one of the mechanisms responsible for AD pathology [173]. Likewise, excitotoxins can activate microglia and stimulate release of inflammatory cytokines and additional glutamate.

Aß has been shown to activate all three MAPK pathways on microglia, thereby resulting in microglial activation and secretion of neurotoxic elements, including glutamate [174]. Further evidence of this interaction between cytokines and excitotoxins comes from the work by Sass *et al*. [175] who demonstrated that Al^{3+} treated astro-

cytes when mixed with neurons caused no degeneration of these cells unless glutamate was added to the culture. It was later that the reason for this effect was elucidated, that is cytokine enhancement of excitotoxicity. It is accepted that IL-1 is neurotoxic, whereas TNF-α can be either neuroprotective or neurotoxic depending on the presence of excess glutamate in a dose and duration-dependent manner. The anti-inflammatory cytokines, IL-10 and TGF-ß appear to be mostly neuroprotective, whereas IL-6 is neuroprotective unless glutamate release has been triggered [176].

Recent studies have shown that fluoride can induce strong IL-8 response in human lung EC by a G-protein activation pathway [68]. Downstream, PKC activation over a prolonged time was observed, which as we have seen in the case of the CNS can activate microglia, resulting in inflammatory cytokine and glutamate release. Again, it is possible that AlFx complex is the operating molecule rather than fluoride alone. An earlier study by Refsnes and coworkers, in which deferoxamine was shown to significantly reduce NaF-induced IL-6 and IL-8 release, strongly indicated that AlFx was indeed the operant molecule in these EC [177].

Also of interest is the finding that focal lesions can produce distant inflammatory injury and that anterograde degeneration was associated with a somewhat later expression of cytokines, whereas retrograde degeneration was associated with earlier cytokine expression in these damaged areas [178]. These observations, when combined with the well demonstrated ability of Al^{3+}, fluoride and AlFx to induce cytokines expression from microglia and astrocytes and at least secondarily increase glutamate release make a strong case for excitotoxicity as an important, if not central mechanism in fluoride neurotoxicity. Likewise, fluoride, Al^{3+} and the AlFx have been shown to dramatically increase brain ROS, RNS and LPO, which has been shown to enhance excitotoxic damage.

Many of these affected reactions involve intracellular Ca^{2+} regulation. It has also been shown that excessive NMDA receptor activation causes an overload of mitochondrial Ca^{2+} with resulting loss of cellular energy levels [179]. The fact that AlFx produces prolonged activation of InsPs and subsequent Ins(1,4,5)P_3 generation, would suggest chronic elevations in $[Ca^{2+}]_i$ with eventual triggering of apoptotic pathways. Nicholls and co-workers [180] found that glutamate elevations in the presence of depressed mitochondrial function resulted in Ca^{2+} dysregulation and an elevation of mitochondrial-generated superoxide. Glutamate excess is also known to increase cellular NO generation, which reacts with the excess superoxide to form the mitochondrial inhibitor peroxynitrite. Also of importance is the observation of Mercocci *et al.* [181] as to the extreme sensitivity of mitochondrial DNA to oxidative stress. The combination of excitotoxicity, oxidative stress, LPO, Ca^{2+} dysregulation and mitochondrial dysfunction greatly increases neuron and glial sensitivity to damage. Toxicity of Al^{3+}, fluoride and AlFx have all been shown to be connected to these various process [101].

The Effects of Fluoride on PPI Signaling Pathways in the Brain

In experiments with brain cortex membranes NaF mimicked the action of GTP(S) in stimulating PPI turnover and generation of InsPs [182, 183]. This effect was highly synergistic with that of $AlCl_3$, supporting the concept that AlFx is the active stimulatory species. Coincubation of submaximal concentration of GTP(S) with AlFx did not result in an additive stimulation of PPI hydrolysis. Paradoxically, $AlCl_3$-induced PPI hydrolysis was potentiated by coincubation with both GDP(S) and phorbolester. Shafer *et al.* [184] compared the effect of 5-30 mM NaF and various concentrations of $AlCl_3$ on muscarinic, adrenergic, and metabotropic receptor-stimulated PPI hydrolysis in cortical and hippocampal slices from rat brain. In agreement with many others, these authors found that NaF stimulates InsPs accumulation as well as the cholinergic agonist carbachol, the adrenergic agonist norepinephrine, and the glutaminergic agonist quisqualate. The higher concentrations of $AlCl_3$ (0.5 mM) inhibited InsPs accumulation stimulated by agonists and that stimulated by NaF.

Tiger *et al.* [41] studied actions of fluoride upon the PPI pathway in the rat brain miniprisms. NaF concentration dependently increased basal PPI breakdown, with a maximum effect being seen at 20 mM. On the other hand, NaF reduced the PPI breakdown responses to stimulation by carbachol, noradrenaline, and serotonin. These authors concluded that fluoride inhibits agonist-stimulated PPI breakdown via actions not only on G proteins but also on PPI-specific PLC substrate availability. The finding that fluoride ions inhibit agonist-stimulated PPI breakdown also on PPI-specific PLC substrate availability was later confirmed [185].

Other Effects

A 2-3-fold stimulation of the basal PLD hydrolytic activity by AlFx in the synaptosomes from canine brain was reported [81, 186]. These results not only indicated that the muscarinic acetylcholine receptor-G protein-regulated PLD is responsible for rapid accumulation of choline and phosphatidic acid (PA) in the brain but also revealed involvement of PLD in a novel, previously unrecognized, signaling pathway in the brain. Kanfer *et al.* [187] used NaF as a PA phosphatase inhibitor to study PLD activity in rat brain cerebral cortical neuronal nuclei. In the absence of NaF the principal product was DAG; whereas in the presence of NaF, the principal product was PA. These authors reported that BeF_2, AlF_3, and PA inhibited the neuronal nuclei PLD activity.

Hypothalamic suprachiasmatic nuclei have been suggested as the site of a biological clock responsible for generation of circadian rhythms. Melatonin receptors are involved in this function [188]. AlFx was used to indicate that the effects of melatonin are mediated by heterotrimeric G protein [189]. AlFx blocked the increase in cAMP stimulation by forskolin, being as effective as melatonin, and increased $[Ca^{2+}]_i$ [190]. The stimulatory effects of AlFx and Ca^{2+} on InsPs accumulation were not additive [191].

When rat hippocampal slices were exposed to 10 mM NaF and 10 μM $AlCl_3$ for a brief period of time (12-15 min), spike amplitude fell to very low levels. Upon washout, spike amplitude recovered beyond control values and in half of the preparations a prolonged enhancement of spike amplitude occurred. If $AlCl_3$ was omitted from fluoride-containing saline, enhancement of spike amplitude, when observed, was brief [192]. Brief bath application of AlFx in standard saline consistently induced a long lasting potentiation in area CA1 of rat hippocampus [193]. NaF (50 μM) and $AlCl_3$ (12.5 μM) were administered alone or in a combination to cultured hippocampal neurons from fetal rats [194]. Al^{3+} affected the development of the interconnecting fibers. This phenomenon was enhanced when NaF was given together with Al^{3+}.

Chen and Penington [195] tested hypotheses concerning the actions of AlFx in the inhibitory effect of G proteins on Ca^{2+} channel activity of dorsal raphe (DR) neurons. These authors suggested that there can be a competition between the receptor and AlFx stimulated G protein activity and investigated whether the interaction occurs at the level of the G protein, or at the level with Ca^{2+} channels. The main findings of this study were that intracellular AlFx caused approximately a one-third of maximum tonic stimulation of the G protein coupled to Ca^{2+} channels of DR neurons, consistent with a G·GDP-AlFx complex resulting in mimicry of the G·GTP complex. These authors suggested a fairly parsimonious explanation of the sequence of events occurring after agonist application to a DR neuron in the presence of AlFx. They proposed that after several applications of 5-hydroxytryptamine, some G proteins are in the basal state and some are activated by AlFx. In summary, their detailed study brought evidence that AlFx modified the OFF-rate kinetics of G protein activation by agonists. Agonist application temporarily reversed the effects of AlFx, making it a comple-

mentary tool to GTP(S) for the study of G protein interactions. The concentration of fluoride used in this study was high (130 mM) in comparison with other laboratory studies. The high excess of fluoride ions could therefore exert many other effects on energy metabolism or protein conformation, including ion channels.

8. Bone Cells

Fluoride becomes the most potent agent inducing uncoupling between bone resorption and formation in favor of formation, thus resulting in an increased bone volume. NaF and AlFx have been shown to be bone cell mitogens [64, 196]. This characteristic of fluoride action on bone together with the observed skeletal fluorosis in human prompted several laboratories to investigate the mechanism, by which fluoride enhances the proliferation and the activity of osteoblastic cells. The high demands for energy are placed on osteoblasts during proliferation, maturation, and production of mineralized matrix. Glycolysis provided ~50% of the energy requirement of mature osteoblasts and is likely to be important for their function [197]. AlFx simultaneously inhibits osteoclastic bone resorption [25, 198].

Osteoblasts

Osteoblasts secrete bone matrix and regulate mineralization process in bone formation. Following the initial observations, that fluoride can directly influence the activity of osteoblastic cells in culture, the effects of fluoride on human osteoblastic cells proliferation were investigated in several laboratories. A first hypothesis for the mechanism by which fluoride enhances cell proliferation has emerged from the observation that osteoblastic acid phosphatase was inhibited in a dose-dependent manner by fluoride. Since fluoride has been known as inhibitor of tyrosin phosphatases, Lau *et al*. [24] suggested that this unique acid phosphatase has tyrosine phosphatase activity in bone-forming cells. In addition to these results, it was shown that fluoride could potentiate the mitogenic action of several growth factors acting through tyrosine kinase membrane receptors.

On the other hand, Caverzasio with co-workers [64] suggested and tested the hypothesis that the mitogenic action of fluoride could involve activation of heterotrimeric G proteins. In their initial studies [82, 199] they noted that the micromolar fluoride concentrations in the presence of traces of Al^{3+} reproducibly enhanced cell proliferation. These observations strongly suggested that AlFx is probably the active fluoride species responsible for the change in bone mineral mass *in vivo*. The sensitivity of the mitogenic effect of AlFx to pertussis toxin suggested a potential role of the G_i protein in mediating this cellular response. Their observations also supported the notion that the change in protein tyrosine phosphorylation, which mediates the proliferative effect of fluoride in bone-forming cells, involves the activation of a tyrosine kinase. With this information, a new mechanism for the enhancement of osteoblastic proliferation by fluoride was proposed.

Two competing models, both of which involve the MAPK mitogenic signal transduction pathways were thus suggested. The first one [49] involves a fluoride inhibition of a unique fluoride-sensitive phosphotyrosine phosphatase in osteoblasts. Such inhibition results in a sustained increase in the tyrosine phosphorylation level of the key signaling proteins of the MAPK pathway, leading to the potentiation of the osteoblastic proliferation initiated by growth factors. A benefit of this model is that it accounts for all the unique properties of the osteogenic action of fluoride. These include the low effective fluoride dose, the requirement of tyrosin kinase-activating growth factors, the sensitivity to changes in medium phosphate concentration, the preference for undifferentiated osteoblasts, and the involvement of the MAPK. The competing model proposes that fluoride acts in coordination with Al^{3+} to form AlFx [64]. This activates a pertussis toxin-sensitive $G_{i/o}$ protein on osteoblast membrane, leading to an activation of cellular protein tyrosine kinases, which in turn leads to increases in the tyrosine phosphorylation of adaptor molecules, activation of the Ras/Raf/ERK pathway, and enhanced cell proliferation.

There is a controversy in these two hypotheses of whether enhancement of tyrosine phosphorylation induced by fluoride results from inhibition of tyrosine phosphatase(s) or activation of tyrosine kinase(s). However, the mechanism by which heterotrimeric G proteins, in particular G_i and G_q, enhance osteoblastic cell proliferation is not completely understood. The mitogenic action of AlFx shows several different characteristics than that of fluoride [49].

Sun with co-workers [200] established a method for isolating and culturing osteoblasts from the newborn rat calvaria. They found that fluoride at low doses promotes the proliferation and differentiation of osteoblasts, whereas at high doses it can induce DNA damage, arrest the cell cycle in S phase, and induce apoptosis. The inhibition of rat osteoblast growth at 10^{-3} M fluoride has been reported [201-203].

Osteoclasts

Osteoclasts function to support calcium homeostasis and to remodel bone. During bone resorption, osteoclasts generate very high $[Ca^{2+}]_e$ in the resorption space. PLC may mediate Ca^{2+}-induced effects in osteoclasts, because Ca^{2+} increases production of $Ins(1,4,5)P_3$ in GCT23 osteoclast-like cells and chicken osteoclasts [204]. Exposure of osteoclasts to AlFx resulted in a marked concentration-dependent inhibition of bone resorption [25, 198]. This inhibition was associated with a dramatic increase of the secretion of an osteoclast-specific enzyme, tartrate-resistant acid phosphatase. Cholera toxin, a G_s stimulator, similarly abolished bone resorption and enhanced acid phosphatase secretion. In contrast, pertussis toxin, a G_i inhibitor, inhibited bone resorption. It seems that AlFx stimulate both PLC and AC in osteoclasts. The osteoclast activity may be influenced by EC via generation of products including PGs, NO, and endothelin.

9. Commentary to Observations of Fluoride Effects on the Cell Level *In Vitro*

Based on the observed effects on enzymatic activities in intact cells, it seems that fluoride easily permeates across the plasma membrane and reaches cytosolic concentration required for its effects as observed on isolated enzymes, homogenates, and membranes. The phenomenological observations of the effects of AlFx on intact cells also indicate that these complexes are, in many cases, appearing in the system after the addition of fluoride and Al^{3+} into the extracellular solution. The slow equilibration kinetics between various compounds of fluoride and Al^{3+} could give rise to puzzling kinetics that also could cause misinterpretation of results. The critical analysis of reported findings does not allow us, in many cases, to conclude whether the observed effects can be accounted to the action of fluoride alone or to its synergistic effect with Al^{3+}. The added Al^{3+} might also react with some non-protein ligands, such as phosphate, citrate, and buffers. Nevertheless, it seems that AlFx exert their effects at very low concentrations. It cannot be excluded that the excess of free fluoride binds further to protein molecules, changing their conformations, stability, and enzymatic activity. The inhibitory effects of fluoride on energy metabolism followed by depletion of ATP [195] and reduction of PLC substrate synthesis [185] could also contribute to the explanation of mechanisms for the observed effects.

G Proteins

The observation that AlFx can activate heterotrimeric G proteins has been useful for the study of G protein involvement in numerous biological systems, for the elucidation of three-dimensional structures of G proteins and several GTPases, for understanding the mechanism of GTP hydrolysis, and the role of GAPs. AlFx can stabilize complexes formed between small G proteins Ras and RhoA and their corresponding GAPs. Nucleotide exchange and

GTP hydrolysis are fundamental to the regulation of all types of G proteins that have been examined to date. G proteins regulate the activities of a structurally diverse group of effectors molecules. These also include enzymes engaged in the synthesis and degradation of intracellular second messengers, as well as ion-selective channels. However, the question of the coordination state of AlFx remains open [88].

PPI Signaling Pathway

The increased breakdown of PIP_2 and the increased production of InsPs have been reported quite often (Table **3**). PLC has been found activated by all classes of cell surface receptors [205].

The regulatory input from G_q-coupled receptors can also control AC activity by Ca^{2+} or PKC-dependent processes. Morris and Malbon [93] explained in their review some paradoxical and unique characteristics of the regulation of the G_q family of heterotrimeric G proteins. Their intrinsic steady-state GTPase activities are much lower than those of members of the other heterotrimeric G protein families but the G_q/PLC- system is activated very rapidly upon addition of an agonist. In this system, receptor-promoted binding of GTP(S) to the G protein and PLC-catalyzed PIP_2 hydrolysis are tightly coupled. When the nonhydrolyzable GTP(S) was replaced by hydrolyzable GTP, PLC activation was much reduced. More detailed studies of the time courses of PIP_2 hydrolysis by PLC-1 in such reconstitution systems suggest that, in the presence of saturating agonist, receptor-G_q complexes can remain stable over multiple GTPase cycles. The binding of AlFx to GDP of G_q might therefore lead to tight coupling of PLC and induce the state of sustained activation.

Physiological Implications

The significant physiological implication brought the observations of additive effect of low fluoride concentrations with an ineffective hormonal agonist resulting in a maximally effective response [39, 64, 69, 74, 138]. On the other hand, NaF reduced the PPI breakdown responses to stimulation by noradrenaline and serotonin. It therefore seems that fluoride might also inhibit agonist-stimulated PPI breakdown via actions on PPI-specific PLC substrate availability [41]. It is evident that interventions of AlFx in a myriad of reactions that involve G proteins have the potential of altering the signaling pathways. The principle of amplification of the initial signal during its conversion into functional response has been a widely accepted tenet in cell physiology. It is evident that AlFx is a molecule giving a false message, which is amplified by processes of signal transduction (Fig. **3**).

Table 3. The Effects of NaF (mM) Plus $AlCl_3$ (μM) on Components of Signaling Pathways

Cells/ Tissue	AC	PLC	InsPs	$[Ca^{2+}]_i$
Hepatocytes	↓↑	↑	↑	↑
Platelets		↑	↑	↑
RBC	↑	↑	↑	↑
Neutrophils		↑		↑
Leucocytes			↑	↑
Fibroblasts	↓	↑	↑	↑
Macrophages		↑		↑
Heart	↑			
Lung cells		↑	↑	
Kidney cells	↑			↑
Neurones	↓↑	↑	↑↓	↑
Astrocytes		↑		↑
Osteoclasts	↑	↑	↑	↑

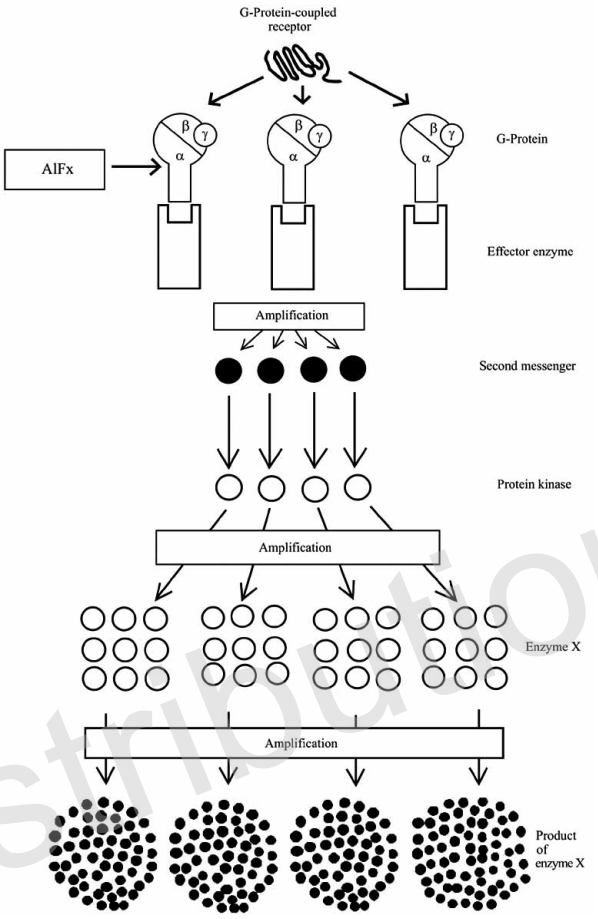

Fig (3). AlFx acts as the messenger of false information. Its message is greatly amplified during the conversion into the functional response of a cell. The second messenger molecule could be cAMP, $Ins(1,4,5)P_3$, and DAG. Moreover, AlFx can participate as the analogue in the phosphoryl-transfer reactions involved in the signaling cascade.

Protein phosphorylation constitutes one of the major posttranslational mechanisms employed in the physiological regulation of G protein-linked signaling. Phosphoryl-transfer reactions are also involved in processes such as regulation of cell metabolism, energy transduction, cytoskeletal protein assembly, regulation of cell differentiation and growth, aging, and apoptosis. Several authors reported the observation of fluoride-induced apoptosis in various tissues. Fluoride and AlFx can induce cell death by the activation of a cell surface receptor and damaging DNA. Understanding cell type specific regulation of apoptosis allows the design of new selective drugs capable of modulating the cellular response [206]. Considering that all these reactions are fundamental for nearly all biological processes, the common denominator of which is the transfer of a phosphoryl group, we can conclude that fluoride, in the presence of trace amount of Al^{3+}, represents a very useful tool for further investigations.

Previous studies have shown PKC to play a central role in glutamate toxicity [154]. Blocking PKC significantly reduced excitotoxic damage *in vitro*. It is also important to point out that the effect of PKC activation is tissue dependent. While in the CNS it enhances glutamate uptake, in EC of human umbilical veins it inhibits glutamate uptake [207]. If it has the same effect on all vascular EC cells, then AlFx and fluoride could increase atherosclerotic changes via glutamate triggered inflammatory eicosanoid generation, excito-

toxicity and an increase in ROS and LPO within vessel walls. This would be especially important in vascular dementia. Glutamate has been shown to dramatically increase LPO products in arterial tissue in adult male mice and to lower all of the major antioxidants in the arterial wall [208]. NaF induced EC barrier dysfunction [209], which was accompanied by the development of actin stress fibers, intercellular gap formation, and significant time-dependent increases in myosin light chain phosphorylation. Stimulation of AA release and PGI2 production by AlFx was observed in cultured EC from rabbit coronary microvessels and cultured pig aortic EC [74, 210].

Refsnes and co-workers demonstrated that NaF powerfully induced IL-6 and IL-8 production and that deferoxamine abolished this response indicating that it was AlFx and not fluoride alone that was responsible for the induction of these cytokines [177]. This implicates a G protein mediated effect. What makes this important is that inflammatory cytokines have been shown to interfere with glutamate transporter protein function. Dysfunctions of glutamate transport play a major role in a number of neurological conditions as well as neurodevelopment. In addition, glutamate transporter proteins have been characterized in a number of tissues outside the CNS, including the placenta, liver, intestine, pancreas, ovary, lung and kidney [211]. These observations, when combined with the well demonstrated ability of Al^{3+}, fluoride and AlFx to induce cytokines expression from microglia and astrocytes and at least secondarily increase glutamate release make a strong case for excitotoxicity as an important, if not central mechanism in fluoride neurotoxicity.

The use of fluoride in laboratory investigations contributed to the discovery of new signaling pathways and their cross talks. Numerous studies of fluoride effects in laboratory investigations from the last decades bring new evidence for our understanding of complicated integrative networks, which regulate the signal transduction processes of the whole organism.

ANIMAL MODELS IN FLUORIDE RESEARCH

The artificial fluoridation of public water supplies at 1-4 ppm in many countries after the Second World War opened the need to study the effects of long-term fluoride intake. A large variety of fluoride concentrations in drinking water was used in animal laboratory studies. For example, rats were fed pure spring water with a natural concentration of 0.2 ppm fluoride or spring water enriched with NaF to a concentration of 0.8, 1.1 or 2.2 ppm fluoride during 180 days exposure [212]. Mullenix *et al*. [213] reported that when rats consumed 75-125 ppm and humans 1-10 ppm fluoride in their respective drinking waters, the result was equivalent ranges of plasma fluoride levels of 0.06-0.64 ppm (2.8-32 $\mu mol.L^{-1}$). The weanling male rat required water fluoride levels of 10 ppm to produce plasma fluoride levels of about 2 $\mu mol.L^{-1}$ [214]. Exposure of rats to 175 ppm resulted in dehydration and the death of 50% of exposed young rat females within 10 days [213]. On the other hand, Ekambaram and Paul [215] studied fluoride effects in adult female Wistar rats after treatment with 500 ppm NaF in drinking water for 60 days. Administration of high doses of fluoride did not produce lethality in this study. Two long-term studies [216, 217] compared the effects of 0.5, 5 or 50 ppm of AlF_3 with comparable levels of NaF in rats. Surprisingly, the highest mortality was observed in the 0.5 ppm AlF_3 group. The administration of NaF alone did not produce a similar mortality rate. The effects of various doses of fluoride are summarized in Table **4**.

1. Systemic Effects of Fluoride

Fluoride when absorbed is rapidly distributed by systemic circulation. Steady-state fluoride concentrations are achieved more rapidly between plasma and well-perfused tissues, such as liver and kidney. The major route for the removal of fluoride from the body is by the kidney [218]. Fluoride in tissues is associated with structural changes and disorders of their function. Laboratory investigations to validate predictive animal models of fluoride or AlFx effects provide evidence that chronic administration of fluoride influence physiological homeostasis in experimental animals. The observed differences might be due to variations in dose, duration of exposure, sensitivity of species, gender, and physiological status of the animals.

The occurrence of hypocalcaemia was reported after fluoride overload of rats and mice [222, 232, 233]. Verma and Sherlin [220] suggested that hypocalcaemia observed in their study might be due to decreased Ca^{2+} absorption from the gut. With a high fluoride intake, insoluble calcium fluoride is formed in the intestine and excreted in faeces increasing the likelihood of low blood Ca^{2+} if there is an insufficient dietary intake. In turn, hypocalcaemia may lead to parathyroid stimulation with a secondary hyperparathyroidism, bone matrix resorption, osteoporosis and osteomalacia [234].

Heard *et al*. [222] reported that the acute fluoride intraperitoneal administration resulted in cardiac dysrhythmias, and cardiovascular

Table 4. Systemic Effects of Fluoride as Observed Under Various Conditions in Animal Models

ANIMALS	DOSE	EXPOSURE	SYSTEMIC EFFECTS	REFERENCES
RATS	0.5 ppm	52 weeks	cell proliferation, nephritis	[216]
	0.8, 1.1 or 2.2 ppm	180 days	$\downarrow Ca^{2+}$, Mg^{2+}, Zn^{2+} in adrenals, myocardium, bone $\uparrow Na^{+}$ in aorta, lung, joint, ECG affected	[212]
	5 and 25 ppm	12 weeks	fluoride accumulation in kidney and liver, increased lipid peroxidation	[218, 219]
	40 mg/ kg of body weight/daily	during pregnancy	dams + F1 generation ↓body weight, ↓serum proteins, hypocalcemia, hypoglycemia, nephrotoxicity	[220, 221]
	175 ppm	10 days	dehydratation, death	[213]
	500 ppm	60 days	↓body weight, ↓serum proteins, hypocalcemia, dental lesions, impaired motor activity	[215]
MICE	57 mg F^{-}/ kg	60 minutes	hypocalcemia, cardiac dysrythmias, cardiovascullar collapse, death	[222]
	10 - 30 mg NaF/kg/day	30 days	hypocalcemia, decrease in fertility, altered sperm structure	[223]
	226 ppm	20 days	↓body weight, renal damage	[224]
PIGS	1.5 mg F/kg/h i.v. infusion	1-3 h	deterioration of cardiopulmonary function	[225]
	100 - 400 mg F/ kg/day	50 days	lassitude, anorexia, sluggishness, lesions in liver, kidney, and thyroid	[226, 227]
RABBITS	5-50 mg NaF/kg/day	105 days	cardiac irregularities, myocardial damage, degenerative and inflammatory changes in the liver, pulmonary damage, functional sterility	[228, 229]
SHEEPS	13.8 ppm	12-24 weeks	↓body weight ,↓serum proteins, dental lesions	[230]
	chronic fluorosis area	long-term	P-Q interval prolonged, sinus bradycardia, decreased heart beats	[231]

collapse in a mouse model. Progressive deterioration of cardiopulmonary function after intravenous infusion of 1.5 mg F^-/kg/h for 3 h (with or without $AlCl_3$) was observed in pigs [225]. At 3 h, mean pulmonary arterial pressure, pulmonary vascular resistance, tracheal pressure, and plasma concentrations of TXB2, 6-ketoPGF1α, and PGF2α were significantly increased to approximately 200, 520, 175, 759, 402, and 336%, respectively, of baseline values. Cardiac irregularities and low blood pressure have been reported in young albino rabbits of both sexes after long-term fluoride administration [229]. The myocardium showed cloudy swellings, sarcoplasmic vacuolization, and small hemorrhages followed by fibrous necrosis. The degree of myocardial damage seemed to be directly proportional to the dosage of fluoride.

Degenerative and inflammatory changes were also observed in the liver of the exposed animals [228]. Histopathological examination revealed increasing degrees of hepatocellular necrosis, hyperplasia, extensive vacuolization in hepatocytes, and centrilobular necrosis in the liver. Experimental fluorosis in rabbits produced pathological lesions in the trachea, pulmonary damage, hypertrophy and hyperplasia in skeletal muscle, and structural alterations in the lens. Pathological lesions in liver, kidney, and thyroid were observed in young pigs exposed to fluoride for 50 days, along with lassitude, anorexia, and sluggishness [226, 227].

Several authors observed that fluoride causes various histological structure changes of the kidney, resulting in impairment of renal function and metabolism. A large number of kidney cells, taken from a group of young pigs after 50 days of fluoride administration, were undergoing or had finished a program of cell death, thus resulting in kidney lesions [226, 227]. These results therefore provide valuable insight on the effects of chronic fluorosis on kidney deterioration.

Isaacson *et al*. [216] compared the effects of 0.5 ppm of AlF_3 with a comparable level of NaF after 52 weeks. No differences were found between the body weights of rats in the different treatment groups although more rats died in the AlF_3 group than in the control group. A progressive decline in the appearance of the AlF_3 animals was noted throughout the experiment, with the hair becoming sparse and the yellowing which occurs with age. The skin became dry, flaky and of a copper color. The kidneys of the AlF_3 group had higher Al^{3+} levels compared to both the control and NaF groups, while liver Al^{3+} levels did not differ between groups. Pathological changes were found in the kidneys of animals in both the AlF_3 and NaF groups. The kidneys from rats drinking the NaF-treated water exhibited glomerular hypercellularity and mesangial proliferation together with patchy focal nephritis. Al^{3+}-containing deposits were found in the kidney blood vessels, and the renal Al^{3+} content was doubled when the rats drank the AlF_3 water. More monocyte infiltration was present in the kidneys of the AlF_3 group compared to the controls. No morphological abnormalities were observed in the liver. Since the administration of NaF alone did not produce a similar mortality rate, this effect does not appear to be directly related to fluoride intake. Both the AlF_3 and NaF groups had increased brain Al^{3+} levels relative to controls. The Al^{3+} level in the NaF group was double that of controls and the Al^{3+} level of the AlF_3 group was even greater.

Fluoride administered at 226 ppm to female mice in drinking water caused thyroid impairment, retarded growth, altered liver, kidney, and bone weights [224]. The plasma level of T_3 and T_4 were lowered by 58%. At the same time, the plasma TSH level were increased 3.15-fold. Hypothyroidism induced by fluoride also affected haematopoiesis. Ge *et al*. [235] suggested that fluoride may directly damage cells and induce rupture of DNA strands and thereby cause dysfunction of the thyroid gland. These authors demonstrated that fluoride can directly induce structural changes and dysfunctions of the thyroid gland of rats. Also, fluoride disturbes the synthesis and secretion of thyroid hormone, interferes with the activity of enzymes that catalyze the conversion of thyroxine (T_4) into the active thyroid hormone triiodothyronine (T_3) and inactive metabolites, thereby leading to perturbations of circulating thyroid hormone levels. DNA strands in thyroid gland cell were adversely affected when rats were exposed to high fluoride, low iodine, and their interactive combination from the age of one month to 20 months. These findings demonstrate that excessive intake and accumulation of fluoride in the body is a serious risk factor for the development of thyroid dysfunction, especially when iodine deficiency also exists.

2. Effects of Fluoride on Embryonic and Fetal Development

Several contradictions have been raised regarding transfer of fluoride through placental barrier. Ream *et al*. [236] reported that the amount of fluoride crossing the placenta is insufficient to produce morphological changes in bones of weanling rats born to dams given 150 ppm of fluoride. On the other hand, various reports have suggested that fluoride crosses the placenta in a number of species including rats, guinea pigs, rabbits, and Holstein cows [237-241].

Collins *et al*. [242, 243] investigated effects of oral administration of 10, 25, 100, 175, and 250 ppm NaF daily throughout gestation in rats and throughout three generations. Decreased fluid consumption observed at 175 and 250 ppm was attributed to decreased palatability and did not affect reproduction. No cumulative effects were observed in three generation. Mating, fertility and survival indices were not affected. The number of corpora lutea, implants, viable fetuses and fetal morphological developments were similar in all groups. NaF up to 250 ppm did not affect reproduction in rats in this study. Oral administration of NaF (20, 40 or 80 mg. kg^{-1} /body weight/day) from day 6 to 19 of gestation in rats significantly lowered body weight gain and feed consumption [244]. No external malformations were observed in any of NaF-treated dams. Increased numbers of resorptions/dead fetuses were observed in 40 and 80 mg NaF-treated rats. Visceral abnormalities such as subcutaneous hemorrhage were also observed.

In another experiment, rats and rabbits were exposed to NaF in drinking water for 10 and 14 days of pregnancy, respectively. The NOAEL for maternal toxicity was 150 ppm for rats and 200 ppm for rabbits based on decreased water consumption and a reduction in maternal body weight gain. No convincing reproductive effects were seen even at the maximum tested concentrations providing doses of 27 mg. kg^{-1} /body weights for rats and 29 mg. kg^{-1} /body weights for rabbits [245]. Trabelsi *et al*. [246] examined the effect of administration of 500 ppm to pregnant and lactating mice from the 15th day of pregnancy to the 14th day after delivery. Compared to a control group, the NaF-treated pups, at age 14days, showed a 35% decrease in body weight, a 75% decrease in plasma free T_4, and reductions in the cerebellar and cerebral protein concentrations by 27% and 17%, respectively. Serum sodium, potassium, and protein concentration increased significantly in the serum of NaF-treated P-generation females and F1-generation rats [221].

Fluoride content in fetal skeleton and teeth increases with the age of the fetus and with the fluoride concentration in the drinking water consumed by the mother. Fluoride is deposited in mineralizing new bone more readily than in existing bone. Bone in which fluoride ions are incorporated is more resistant to bone remodeling and thus lead to a more brittle skeleton as it ages [247]. An increase in incidence of skeletal abnormalities, such as presence of 14 ribs, wavy ribs, dumbbell shaped 6th sternebrae, and incomplete skull ossification, were observed in fetuses after oral administration of various doses of NaF from day 6 to 19 of gestation in rats [244]. A significant increase was seen in rats in average number of fetuses with three or more skeletal variation in the 250 ppm (25.1 mg/kg body weight) group [238, 243]. Ossification of the hyoid bone of F2 fetuses was significantly decreased. Fluoride has been reported to

decrease the bone quality of femoral shaft and neck in 30 and 60 mg $F^-.L^{-1}$ treated young growing rats 6 weeks of age [248].

The potent teratogenicity of fluoride was demonstrated *in vitro* on embryo limb bud cells of both rat (13-day) and mouse (12-day), which were subjected to culture for 5 days [42]. Fluoride inhibited cell differentiation (ID_{50} 6.8 $\mu g.ml^{-1}$ for rat, 7.3 $\mu g.ml^{-1}$ for mouse) and proliferation (ID_{50} 44.1 $\mu g.ml^{-1}$ for rat, 63.6 $\mu g.ml^{-1}$ for mouse). The ability of NaF to induce changes in the development was also studied in frog *Xenopus* embryo for NaF concentrations ranging from 0 to 200 ppm. The minimum concentration to inhibit growth was found to be 140 ppm [249]. The reduction in the head-tail lengths of tadpoles by NaF, the pigmentation, the eye diameters, and the touch reflex of embryos were observed. Immobility has been linked to defects in the neuromuscular system of tadpoles. Neuromuscular developmental defects and effects on the brain and behavior were also demonstrated in newborn rats when pregnant dams were treated with fluoride in the drinking water [213, 217].

3. Brain and Behavior

During the last decade numerous animal studies have been published, which have raised the level of concern about the impacts of increasing fluoride exposure on the brain. A considerable amount of research has accumulated indicating that fluoride/AlFx can adversely affect the brain in a number of animal species. A decrease in learning abilities and altered behavior, poor motor coordination, loss of neuronal and cerebrovascular integrity, changes in brain membrane lipids and oxidative stress by fluoride alone or in synergy with Al^{3+} in animals drinking fluoridated water have been reported [217, 250-254]. Malondialdehyde, the marker for the extent of LPO, was elevated in the brain of rats treated with 100 ppm fluoride. Also levels of total glutathione, GSH, and ascorbic acid were decreased. Increased oxidative stress could be the mediating factor in the pathogenesis of fluoride toxicity in the brain of the young rats [104, 255].

The observed effects were dependent on the age at exposure to fluoride. The fetal blood-brain barrier is immature and readily permeable to fluoride [256]. Mullenix *et al*. [213] reported the first laboratory study, which demonstrated *in vivo* that the CNS function was vulnerable to fluoride. The accumulations of fluoride were found in all the regions of the brain, with the highest levels in the hippocampus, one of the most sensitive area of the brain to neurotoxicity.

Behavioral Changes

The behaviors identified by the computer consisted of five major body position (stand, sit, rear, walk, and lying down) and eight modifiers (groom, head turn, look, smell, sniff, turn, wash face, and blank) were tested in pups and adult animals of both sexes. Experimental dams received subcutaneous injection of 0.13 mg $.kg^{-1}$ NaF to produce peak plasma fluoride levels of 0.15-0.20 ppm (0.79-1 μM) [213]. This level corresponds to the inorganic fluoride level in human blood in areas with 1 ppm of fluoride in drinking water. Beyond the prenatal period, these pups received no other experimental fluoride treatment. Male and female adult rats were given 100 ppm fluoride in drinking water for 6 weeks (to reach the plasma fluoride level comparable with humans). The behavioral changes common to weanling and adult exposures were different from those after prenatal exposure. Whereas the prenatal exposure to NaF via the mother induced many behaviors in the pup such as drug-induced hyperactivity, weanling and adult exposures led to behavior-specific changes more related to cognitive deficits. When fluoride exposure began at 21 days of age, a common pattern among behavior disturbances developed in both sexes. Adult exposure was associated with significant behavioral impact only in females. Adult males did not have significantly elevated fluoride levels in the hippocampus. This study has gained support from other animal studies [251, 253, 257]. While these studies have employed different methods and animals, they are consistent in that fluoride exposure may impact behavior and/or learning. Also the interactive effects of high fluoride concentration and iodine deficiency might affect the functions of the CNS. Such combination has a negative effect on learning-memory of offspring rats [258]. In addition, even mild hypothyroidsm of the mother during gestation has been shown to lead to significant and permanent defects in brain development in the offspring in both experimental animals and humans.

Neuropathological Changes

Some authors suggested that the effects on behavior were consistent with interrupted hippocampal development histology. Prenatal exposure on 17-19 days of gestation in the rat is a period when pyramidal cells of hippocampus are forming and granule cells of the dentate gyrus of hippocampus form at the ages when weanling and adult exposures were administered. Histological changes were found in the cerebellum of the pups after exposure of pregnant and lactating mice from the 15th day of pregnancy to the 14th day after delivery to NaF [246]. The external granular layer was markedly reduced or absent, the Purkinje cell bodies were poorly differentiated and arranged in a single layer at the surface of the internal granular layer. More apoptotic Purkinje cells were observed.

Neuropathological changes were found in brain tissues from albino rabbits after fluoride subcutaneous injections in different doses to rabbits of both sexes for 100 days [259]. Tremors, seizures, and paralysis indicating brain dysfunction were seen. The Purkinje neurons exhibited chromatolysis and acquired a "ballooned" appearance. Nissl substance showed various degrees of decrease and even complete loss. A reduction in unmyelinated nerve fibres, external granular layer of cerebellum and increased neuronal apoptosis have also been reported in rats and mice [246, 260]. All of these changes are seen with excitotoxicity.

Chronic exposure to fluoride (0.5 ppm for 52 weeks) in drinking water of rats compromised neuronal (hippocampal) and cerebrovascular integrity [216, 217]. These studies were undertaken to compare 0.5 ppm of AlF_3, with a comparable level of fluoride administered alone in the form of NaF. The effects of the two treatments on cerebrovascular and neuronal integrity were qualitatively and quantitatively different with the alterations being greater in animals in the AlF_3 group than in the NaF group, and greater in the NaF group than in the controls.

In the hippocampus, more moderately damaged and grossly abnormal cells were present in areas of the right hippocampus of both the AlF_3 and NaF groups than in the control group. The right hippocampus also had higher levels of Al^{3+}-induced fluorescence than the left hippocampus. The reduction of neuronal density in the neocortex of the left hemisphere was more prominent in the AlF_3 group than the NaF and control groups. Cellular abnormalities in the form of chromatin clumping, enhanced protein staining, pyknosis, vacuolation, and the presence of ghost-like cells were also more common in the AlF_3 group in the left hemisphere. Striking parallels were seen between Al^{3+}-induced alterations in cerebrovasculature and those associated with AD and other forms of dementia. The AlF_3 group had more immunoreactivity for Aβ in the lateral posterior thalamic areas of both hemispheres relative to the controls. The NaF group differed from the control for immunoreactivity for Aβ in the right lateral posterior thalamic area with the controls having low reactivity and the NaF group having no or high levels of immunoreactivity. While the small amount of AlF_3 (0.5 ppm) in the drinking water of rats required for neurotoxic effects was seen as surprising, the neurotoxic effects of NaF (1 or 2.1 ppm of fluoride) was seen as even more so. In summary, the chronic administration of AlF_3 and NaF in the drinking water of rats resulted in distinct morphological alterations in the brain, including effects on neurones and the cerebrovasculature. Many of these pathological changes could be due to either secondary or primary excitotoxicity [101].

Further studies are needed to establish the relative importance of a variety of potential mechanisms contributing to the observed effects of fluoride, Al^{3+}, and AlFx in the brain as well as to determine the potential involvement of these agents in neurodegenerative diseases. In addition, studies need to be done on the effects of fluoride and AlFx on microglial activation, glutamate transport proteins and brain extracellular glutamate levels in response to fluoride/AlFx toxicity. It is also known that excitotoxicity induces brain calcification micro-deposits, which could be a nidus for fluoride accumulation in high concentrations. Studies are needed which measure fluoride levels in brain calcifications in humans, such as basal ganglion calcifications.

4. Paradoxical Dose-Response Effects of Fluoride in Animal Studies

The review of laboratory studies on the physiological and biochemical effects of fluoride reveals the existence of paradoxical dose-responses. Some of them show that, under certain circumstances, the inhibitory or stimulatory impact of fluoride can actually be greater at a lower level of intake than at a higher level (hormesis effect) [261]. Messer *et al*. [262] reported that low levels of fluoride in food rendered mice infertile while a high fluoride diet improved their fertility. Mullenix with co-workers [213] found that the six weeks of consuming drinking water with 75 and 100 ppm fluoride produced higher plasma fluoride levels than did 125 ppm in rats. Bohatyrewicz *et al*. [248] recorded higher compressive bone strength after six weeks in rats drinking water with 8 ppm than with 30 or 60 ppm. The hormesis effect of fluoride has been demonstrated during the initial bone-forming stage in rat skull-cap bone [200]. Fluoride at low doses promotes the proliferation and differentiation os calvarial osteoblasts, whereas at high doses affects the cell structure and cell cycle by rendering the cell stagnant in the S phase and inducing apoptosis. While a significant reduction in the content of ATP in RBC was found in rats after 4 weeks of exposure to 4 or 16 ppm NaF, after 8 weeks, the ADP content remained significantly reduced with the smaller dose, while the greater dose was surprisingly associated with a higher energy potential of the cells [121].

The *in vivo* study of brain LPO and antioxidant systems of young rats in chronic fluoride intoxication revealed that an overriding adaptive response appeared to be operating at the higher fluoride intake [104]. Young rats were exposed to 30 ppm or 100 ppm fluoride in their drinking water for 10 weeks after birth. Malondialdehyde as a marker of LPO was elevated in the young rats exposed to 100 ppm but not to 30 ppm dose. On the other hand, levels of total glutathione, reduced glutathione, and ascorbic acid were elevated in the rats exposed to 30 ppm but were lower in the 100 ppm group. The elevation of glutathione S-transferase activity compared to the controls was much greater in the 30 ppm rats (143% higher) than in the 100 ppm group (21% higher). Such reactions are common. In the lower dose, the cells have time to upregulate protective glutathione and glutathione S-transferase levels, but at the higher dose the mechanism is quickly overwhelmed.

An impressive illustration of this fact is seen in the administration of AlF_3 to rats. In both a 45-week study and a confirmatory 52-week study [216, 217] the neuronal, cerebrovascular, and nephritic toxicity of AlF_3 at 0.5 ppm in the drinking water was significantly greater than with higher levels of AlF_3 (5 or 50 ppm). It is unclear why higher levels of AlF_3 produced less impairment, fewer deaths and generally healthier animals than the low levels. The authors suggested the possibility that fluoride, at certain low levels, may exert a protective effect against the Al^{3+} when given at a certain absolute level.

HUMAN EXPOSURE TO FLUORIDE

In 1942, H. Trendley Dean published his famous 21 City study in which he showed that at 1 ppm fluoride there was a marked decrease in tooth decay [7]. The artificial fluoridation of drinking water as a way of preventing dental caries has been a practice for many years in several countries. Recently, we have witnessed that a growing majority of countries do not support water fluoridation. Today, approximately 60-70% of the American people and more than 50% of the population of Australia, Columbia, Ireland, New Zealand, and Singapore are supplied with fluoridated drinking water. However, people now get fluoride from many other sources, such as food and beverages, pesticide and fertilizers, industry, dental treatments, fluorinated drugs, and fluoride air pollution, so total fluoride intake has become an issue of particular concern [9, 10]. The problem of high fluoride in ground water is one of the most important health-related environmental issues in India. Endemic fluorosis occurs in many parts of China, where the major sources are ground water, coal-burning and brick-tea. In central and northern Mexico millions of people are affected by high fluoride content in household-use groundwater [263].

Numerous epidemiological and clinical studies demonstrate the positive correlation between the higher intake of fluoride and various non-specific symptoms, changes in teeth and bone structure, reduction of children intelligence, and psychiatric symptoms in adults. Most of the ill effects caused by fluoride were first recognized among workers in aluminum factories, where fluoride and Al^{3+} are present in high concentrations [264, 265]. It has become apparent that dental and skeletal fluorosis impact millions of people in fluoridated communities and in countries with regions of high endemic levels of fluoride, like India and China.

1. Fluoride Levels in Human Body Fluids

Under most conditions, fluoride is rapidly and extensively absorbed from the gastrointestinal tract. The rate of gastric absorption is inversely related to the pH of the gastric contents. High concentrations of Ca^{2+} and Al^{3+} can reduce the uptake of fluoride at this stage and the complexes or insoluble fluoride usually exit the body in the feces. Fluoride removal from plasma occurs by calcified tissue uptake and urinary excretion. About 99% of the body burden of fluoride is associated with calcified tissues, and most of it is not exchangeable. In general, the clearance of fluoride from plasma by the skeleton is inversely related to the stage of skeletal development [266]. In a healthy adult, about 50% of the fluoride, which enters plasma, is excreted by the kidney. The estimation of fluoride concentration in human body fluids has been widely performed, since the levels of fluoride in the serum, plasma or urine are important determinants of fluoride effects in the body. However, numerous data are expressed in various units, authors are using various methodologies, and wide variations in fluoride content might exist within various areas and within the same community.

Plasma Fluoride Level

Pak *et al*. [267] suggested the "therapeutic window"of fluoride level in blood to be 5 $\mu mol.L^{-1}$ (95 ppb). Concentrations of fluoride up to 1-2 $\mu mol.L^{-1}$ are not assumed to be overtly cytotoxic. The mean plasma fluoride levels in healthy subjects of 0.68-3 $\mu mol.L^{-1}$ (13 - 57 ppb) were reported [268, 269] in nonfluoridated areas. Table **5** shows selected examples of the average fluoride concentrations in human blood serum and body fluids. The comparison of fluoride level in serum of children living in areas with low fluoride level (0.30 ppm) in drinking water shows the lowest values for 8-16 year old students from Switzerland [270], while in China the estimated serum level was higher [271, 272]. In the group of 21 children living in areas of Delhi, India, with comparable levels of fluoride in drinking water, the mean value of serum fluoride content was 7.37 $\mu mol.L^{-1}$ (140 ppb). Only 4 children had serum fluoride content 1 $\mu mol.L^{-1}$ (19 ppb), the remaining had elevated levels up to 15.8 $\mu mol.L^{-1}$ (300 ppb) [273].

The US Environmental Protection Agency currently consideres safe levels of fluoride in drinking water ≤ 4 ppm. Children from the

Table 5. Fluoride Concentration in Human Body Fluids (Areas <1 ppm, Without Fluoride Supplementation)

BODY FLUID	ppb	µmol L^{-1}	REFERENCES
Blood serum			
Europe	13 - 57	0. 68 - 3	[268, 270]
China	41	2.16	[271, 272]
India	19 - 300	1 - 15.8	[273]
Saliva	19 - 59.5	1 - 3.13	[274, 275]
Urine			
Europe	245 - 615	12.89 - 32.6	[270, 275]
China	700 - 830	36.84 - 44.37	[276]
Cord plasma	28	1.42	[277-280]
Amniotic fluid	10 - 17	0.53 - 0.89	[277]
Mother´s milk	5 - 10	0.26 – 0.53	[281, 282]

high fluoride village in China (2.45 ppm) had serum fluoride level 4.26 µmol.L^{-1} (81 ppb) [271, 272], while in children from area in India with 4.37 ppm in drinking water their serum ranged from 1 to 21.6 µmol.L^{-1} (19-410 ppb) [273].

Fluoride in Saliva and Urine

Oliveby *et al*. [274] reported the normal concentration of fluoride in saliva about 1 µmol.L^{-1}. Tóth *et al*. [275] estimated fluoride levels in saliva in a group of 79 subjects of both genders in the age from 19-45 years under experimental design with no fluoride from food chain. The average baseline fluoride concentration in saliva calculated from these published data was 3.13 µmol.L^{-1} (59.5 ppb). This increased after four week test period with daily intake of 1 mg of fluoride from fluoridated salt, milk, and tablets to 19, 32, and 30 µmol.L^{-1} (360, 610, and 570 ppb), respectively. Salivary fluoride concentrations peak rapidly (1 to 15 min) after ingestion but the return to baseline takes 20 to 60 min.

The concentration of fluoride in urine is higher in comparison with the serum and saliva. It is very difficult to compare the results in various studies since various methods have been used. The values given for fluoride concentration in urine from unpolluted areas in the Europe [270, 275] are lower than that reported from China. Analysis of the literature data of Chinese populations in different geographical regions without fluorosis [276] demonstrate a mean urinary fluoride content of 36.84 µmol.L^{-1} (700 ppb) for children and 43.7 µmol.L^{-1} (830 ppb) for adults. Surprisingly high urinary fluoride was found in children in Gdańsk, Poland [283]. Fluoride has been determined in urine of 1 240 children, aged 7-14. Their schools are located near a fluoride-bearing phosphate fertilizer waste disposal site or near a phosphate fertilizer plant. The mean fluoride concentration in urine of 992 children from areas with low fluoride in drinking water (0.2-05 ppm) was 919 ppb (210 – 5 240), while in areas with 1-2 ppm the mean concentration 1 800 ppb (500-6 000ppb) was found. Significantly higher urinary fluoride concentrations were found in boys than in girls.

Fluoride Level in Pregnancy

Although promoted now for some years, prenatal systemic administration of fluoride supplements to pregnant women for caries prevention in their offspring has continued to be controversial. The fasting morning urine levels of 31 pregnant women aged 22–34 living in Poznan, Poland, where the level of fluoride in drinking water ranges from 0.4 to 0.8 ppm were 653 ppb for women in their 28th week and 838 ppb in their 33rd week of pregnancy [284]. The difference of fluoride concentrations in urine samples of the study and the control group of non-pregnant women (835–2 221 ppb) may be explained by incorporation of fluoride into fetal hard tissues and, accordingly, decreased elimination in the urine. This fact must be remembered when evaluating fluoride exposure in women who are pregnant. The statistically significant increase in urine fluoride concentrations observed in the 33rd week of pregnancy suggests that fluoride metabolism is changing with the progress of pregnancy. This fact might be connected with the lower uptake of fluoride in fetal calcified tissues and decreased bone calcification toward the end of pregnancy.

Fluoride has been found in fetal cord blood at various stages of normal pregnancies, from an area with a relatively low water fluoride content (less than 0.5 ppm). Chlubek *et al*. [278] reported that maternal and cord plasma did not differ significantly (33 and 28 ppb, respectively), while Gupta *et al*. [280] found that average fluoride concentration in the cord blood was 60% of that in mother's blood. A significant difference between the cord plasma fluoride levels of the newborns in the untreated group of women (27 ppb) and the fluoride- supplemented groups (58 ppb) was found [279]. Amniotic fluid fluoride levels were significantly higher at term than in midtrimester pregnancy (17 vs 10 ppb). This higher concentration may imply higher fetal urinary excretion of fluoride at term [277].

Mother´s Milk

The very low level of fluoride (5 – 10 ppb) present in mother's milk is probably the evidence that fluoride is not an "essential nutrient" [281, 282] and it is only moderately increased with substantially greater fluoride intake by the mother. Even at the very low normal level, breastfed babies excrete more fluoride than they ingest from the milk. The body usually retains trace minerals that have a genuine physiological role rather tenaciously.

2. Dental and Skeletal Fluorosis

Neither dental fluorosis (DF) nor skeletal fluorosis (SF) are the specific topic of this review. Nevertheless, DF is the first evident sign of increased fluoride intake and several studies bring correlation between the prevalence of DF and other variables studied. Teeth are the first tissue, which shows the excess of fluoride. SF is the end result of the long-term exposure to chronic fluoride intake. The mean urinary fluoride, which can serve as an indicator for identifying endemic fluorosis areas are 84.21 µmol.L^{-1} (1.6 ppm) for adults and 73.68 µmol.L^{-1} (1.4 ppm) for children [276]. Levels of 310 µmol.L^{-1} (5.9 ppm) fluoride were found in 80´s in endemic fluorosis areas in China [285].

Dental Fluorosis

The compilation of studies worldwide indicates that somewhere between 13.5% and 48% of children in fluoridated communities has DF [286, 287]. The overall prevalence of DF is higher in endemic areas. In Rajastan, India, 76.9% of examined children exhibited DF [288] and 84 % of resident children were affected with DF in Haryana, India [289]. DF was detected in more than 80% of the population in endemic areas in Mexico [263]. Moderate and severe DF is associated with negative psychological effects on those afflicted.

Skeletal Fluorosis

Fluoride causes paralysis of limbs in advanced SF, which is related to pressure upon the spinal cord by newly formed bone protruding into it and also upon nerves at the point of their exit from the spine. The spinal cord lesions and muscular damage in patients suffering from SF are also the result of direct action of fluoride on ganglion and muscle cells. Crippling SF was found at and above 2.8 ppm fluoride in drinking water in villages in India with a prevalence of 38%. A prevalence of 31% was found in Wamiaho village (China) with 2.45 ppm fluoride in drinking water [271]. In a group of 1998 subjects above 21 years examined in central Rajastan, SF was diagnosed in 47.5% [288]. A positive significant dose-response relationship between the serum fluoride concentration and the prevalence of SF was demonstrated. However, extremely polluted areas exist in China, where the daily fluoride intake might reach 6.57 and 8.54 mg. The prevalence of SF was 44.4% and 95%, respectively [290].

3. Nephrotoxicity

High fluoride concentrations in drinking water in endemic areas are known to cause impaired kidney function involving renal tubular and glomerular dysfunctions [291, 292]. The fluoride excretion is reduced and fluoride accumulates in the body. In people with kidney disease, the distribution of fluoride in the body fluids and tissues can change dramatically, with less fluoride excreted and more incorporated in mineralized tissues and more remaining in the plasma. As a result, people with kidney disease in areas with 1 ppm fluoride have been found to have significantly elevated bone and serum fluoride levels (up to 19 $\mu mol.L^{-1}$ ~360 ppb) [293]. Fluoride intoxication has been described in chronic hemodialysis patients [294, 295]. Arnow *et al*. [296] reported that 12 of 15 patients receiving dialysis treatment in one room became acutely ill, with severe pruritus, multiple nonspecific symptoms, and/or fatal ventricular fibrillation. Death was associated with longer hemodialysis time and increased age compared with other patients who became ill. The source of fluoride was the temporary deionization system used to purify water for hemodialysis. In some regions, the water used for the dialysate also contained a lot of Al^{3+}. Some patients used Al^{3+}-containing medications. Moreover, patients with renal failure cannot remove Al^{3+} from the blood. Elevated Al^{3+} levels have been also implicated as the cause of dialysis encephalopathy or dementia [297, 298]. Although flurane anesthetics may produce plasma fluoride concentrations in excess of 50 $\mu mol.L^{-1}$ (950 ppb), they have not produced the acute nephrotoxic effects [299]. It seems that the human body has efficient homeostatic mechanism to respond to short time peak of fluoride in the blood.

4. Central Nervous System

In light of the published findings, the long-term synergistic action of fluoride and AlFx represents a risk factor for the functioning of the CNS. G protein-coupled receptors have key roles in information processes in the brain. A number of conditions can trigger excitotoxicity and increase excitotoxic sensitivity including hypoxia/ischemia, depressed cellular energy production from any cause, hypomagnesmia, inflammatory cytokines and eicosanoids, free radical and LPO products, trauma, certain heavy metals, viral, bacteria and fungal infections. This means that a large number of people fall within a hypersensitive state to excitotoxicity. Under such conditions, even physiological levels of extracellular glutamate can be neurotoxic.

Fluoride Exposure and IQ Deficits in Children

While we know that fluoride might cross the placenta, we know little of its impact on the human fetal brain. A study by Du [300] revealed adverse effects on the brains of 15 aborted fetuses between the 5-8th months of gestation from an endemic fluorosis area in China compared with those from a non-endemic area. Stereological study of the brains showed that the numerical density of the volume of the neurons and the undifferentiated neuroblasts as well as the nucleus-cytoplasm ratio of the neurons was increased. The mean volume of the neurons was reduced. These results showed that chronic fluoride overload in the course of intrauterine fetal life may produce certain harmful effects on the developing brain of the fetus. This could represent fluoride/AlFx alterations in cerebral glutamate levels, which are known to play a vital role in neuron migration and pruning of synaptic connections and dendrites.

Several studies appeared from China, which indicated a lowering of IQ associated with fluoride exposure [301-303]. Their conclusions have been criticized because of the possibility of unaccounted confounding variables. However, the latest study by Xiang *et al*. [304] controlled for parental economic status and education, as well as exposure to iodide and lead. These authors found that IQ scoress below 80 were significantly associated with higher serum fluoride level and estimated that children's IQ would be lowered at 1.8 ppm fluoride in drinking water. Such a finding represents little margin of safety considering the potentially serious outcome for infants drinking fluoridated water.

Psychiatric and Mental Disturbances in Adults

A distinct decline in mental activity, poorer memory, inability to coordinate thoughts and reduced ability to write were observed in aluminum smelter workers and persons living near the factory [265, 305, 306]. In light of the published findings, the long-term synergistic action of fluoride plus Al^{3+} represents a hidden but serious and powerful risk factor for the development of AD. Laboratory investigations bring evidence that AlFx may induce and affect all pathological hallmarks of AD [6]. The etiopathology of AD might serve as the example of AlFx long-term action from affecting molecules to the development of this devastating disease. Fluoride alone or in synergistic action with Al^{3+} affects processes of neurotransmission, generation of second messengers, and Ca^{2+} homeostasis. It has been demonstrated that AlFx induces toxic Aß generation, protein τ phosphorylation, and alterations in cytoskeletal protein organization. Amyloid plaques in AD contain Al^{3+} [307]. Many neurodegenerative disorders are generally accepted to stem from pathological changes in the conformation of proteins and thus, are characterized by the accumulation of extracellular and/or intracellular protein aggregates [308]. Moreover, the disturbances of energy metabolism can contribute to the development of neurodegenerative diseases, primarily by enhancing excitotoxic sensitivity and microglial activation. $[Ca^{2+}]_i$ affects many of these processes. Ca^{2+} homeostasis in the cell is also known to deteriorate with aging [309], making the elderly more vulnerable to both excitotoxicity and fluoride/AlFx toxicity.

Suppressed activity of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complex (KGDHC) was found in a number of neurodegenerative diseases, particularly AD and Parkinson's disease [310]. Inhibition of KGDHC has also been shown to alter intracellular Ca^{2+} regulation, something common in neurodegeneration [311]. Gibson *et al*. [310] found that patients with AD carrying the ε4 allele of apolipoprotein E (ApoE4) demonstrated a stronger correlation between their dementia scores and KGDHC activity than did those with non-ApoE4 genes. While there is no direct evidence that fluoride or AlFx complex can suppress KGDHC activity, it is known to be very sensitive to free radical and LPO suppression [312]. That fluoride and Al^{3+} have been shown in a number of studies, both *in vitro* and *in vivo*, to trigger the formation of ROS and LPO products has been well demonstrated. Mercocci *et al*. [181] found that oxidative mtDNA damage was 10-fold higher in mtDNA than nuclear DNA and 15-fold higher in samples taken from individuals older than 70 years. The study was done in individuals aged 42 to 97 years without neurological disease or injury. The combination of excitotoxicity, oxidative stress, LPO, Ca^{2+} dysregulation and mitochondrial dysfunction greatly increases neuron and glial sensitivity to damage.

5. Endocrine Glands

Understanding the role of G proteins in cell signaling allows us to accept the fact that fluoride entering the human body from the environment, water, and food chains followed by Al^{3+}, can affect the activity of endocrine glands and the processes of hormonal regulation of the human body.

Pineal Gland

Luke [313, 314] reported that fluoride accumulates in the human pineal gland. When Luke had the pineal glands from 11 human corpses analyzed, the fluoride in the apatite crystals averaged about 9,000 ppm and in one case went as high as 21,000 ppm. The pineal gland is considered to be a transducer of photoperiodical information [315, 316]. Production of the both chief pineal hormones serotonin and melatonin is cyclical and influenced by light. Melatonin is responsible for regulating numerous life processes, including development and aging. Mongolian gerbils fed higher doses of fluoride excreted less melatonin metabolite in their urine and took a shorter time to reach puberty. In the light of these findings it is interesting to note that the Newburgh-Kingston fluoridation trial (1945-55) found that the girls in fluoridated Newburgh were menstruating on average 5 months earlier than girls in unfluoridated Kingston [317]. Considering the importance of hormonal production of the pineal gland, this issue warrants further study. It is also known that production of melatonin by the pineal is controlled by a metabotropic glutamate receptor and that excess aspartate or glutamate activity can inhibit melatonin release. Being a G protein type receptor, AlFx could also activate this receptor.

Thyroid

Up until the late 1950's, the doses of fluoride as low as 2.3 - 4.5 mg/day were recommended in Europe to reduce the activity of the thyroid gland of those suffering from hyperthyroidism [318]. The search for a mechanism to explain how fluoride might lower thyroid activity has a very long and elusive history. A promising hypothesis is that fluoride mimics the thyroid-stimulating hormone (TSH) by switching on its associated G protein. However, this is puzzling because this would suggest that fluoride would stimulate thyroid activity, not lower it. A possible explanation has come from Tezelman *et al*. [319] who have suggested that overproduction of cAMP leads to a feedback mechanism resulting in a desensitization of the TSH receptor, thus ultimately leading to reduced activity of the gland. It was shown that normal healthy individuals had thyroid function lowered when consuming water at 2.3 ppm [320]. The thyroid gland appears to be the most sensitive tissue in the body to fluoride burden, which is able to increase the concentration of TSH and decrease the concentration of T_3 and T_4 hormones, thereby producing hypothyroidism [258].

Dysturbance of thyroid hormone production has been found in correlation with lowered IQ in children in China [304]. A decreased level of thyroid hormone T_3 was found in residents of Villa Ahumada, Mexico, where fluoride concentration in drinking water averages 5.3 ppm [321]. The first study, which investigated the production of thyroid hormones and TSH, included 90 children living in fluoride endemic, non-iodine deficient areas of Delhi, India, along with 21 children from non-endemic areas [273]. The received data indicate the association of excess of fluoride intake and thyroid hormone disturbances leading to manifestation of iodine deficiency disorders (IDD). This study clearly documents that the primary cause of IDD may not always be iodine deficiency, but the excess of fluoride might induce it. Susheela suggests that iodine metabolism is being disturbed through the fluoride effect on deiodinases, the three enzymes, which regulate the conversion of T_4 to T_3 in target tissues. Even in some of the children from the control group consuming water < 1 ppm F, fluoride levels in their blood and urine are above current upper limits. This indicates other sources of fluoride, such as food and beverages, dental products, air, etc. In those children disturbances in thyroid hormone levels were observed as well. The role of excess of fluoride in development of IDD has been largely unnoticed at present, despite the fact that millions of children suffer with IDD. Considering the globally increasing problem of IDD that issue needs to be taken into consideration.

Testes

The comparison with healthy males living in areas nonendemic for fluorosis suggest that fluoride toxicity may cause adverse effects in the reproductive system of males living in fluorosis endemic areas [322, 323]. A reduction in the circulating testosterone level of males was found in males with or without the presence of clinical SF. The authors concluded that a fluoride exposure of 3–27 mg/day induces a subclinical reproductive effect that can be explained by a fluoride-induced toxic effect in both Sertoli cells and gonadotrophs. A significant decrease in fertility in 30 regions spread over 9 U. S. states with 3 ppm fluoride or more in the water was also reported [324]. Most regions showed an association of decreasing total fertility rate with increasing fluoride levels.

AMELIORATION OF FLUORIDE PATHOPHYSIOLOGICAL EFFECTS

Laboratory studies have revealed that withdrawal of fluoride resulted in some recovery. Withdrawal of fluoride during lactation caused significant recovery in serum changes in both P- and F1-generation rats [220, 221]. Complete recovery from fluoride toxicity in reproductive functions in male mice on co-treatment with vitamins E and D alone and in combination was reported [233]. Ameliorative effect of these vitamins in NaF-treated dams could be due to removal of cell damaging free radicals. Recovery was also possible by feeding antioxidants (superoxide dismutase, glutathione, β-carotene, and some herbal extracts) [102]. Liu *et al*. [325] reported that synthetic catalytic scavengers of ROS proved beneficial in mouse brain for reversal of age related learning deficits and oxidative stress in mice.

Vitamins C and E act as antioxidants scavengers of free radicals and peroxides, which accumulate after fluoride exposure. Vitamin E channels the conversion of oxidized glutathione to reduced glutathione, which in turn helps compression of mono- and dehydroascorbic acid to maintain ascorbic acid levels. Oral administration of vitamin C (50 mg/kg body weight/day) and vitamin E (2 mg/0.2 ml olive oil/animal/day) from day 6 to 19 of gestation along with NaF (40 mg/kg body weight) significantly ameliorates NaF-induced total percentage of skeletal and visceral abnormalities. Vitamin E was comparatively less effective than vitamin C [326]. Vitamin D is known to promote intestinal absorption of Ca^{2+} and phosphate. Cotreatment with vitamins C, D, and E ameliorates NaF-induced reduction in serum Ca^{2+} and phosphorus [327]. Ekambaram and Paul [215] reported that calcium carbonate prevents not only fluoride-induced hypocalcemia but also the locomotor behavioral and dental toxicities of fluoride by decreasing bioavailability of fluoride in rats. Toxic effects of fluoride were reversible if its exposure was withdrawn for 2 months. Intraarterial administration of 1.8 mM $CaCl_2.kg^{-1}$ reduced the risk of death by 33% in a mouse lethal model of fluoride poisoning [222].

Poor nutrition is seen to be an important cause of endemic osteomalacia in high fluoride areas. Reversal of fluoride induced cell injury and fluorosis through the elimination of fluoride and consumption of a diet containing essential nutrients and antioxidants have been shown [328]. Increasing dietary proteins, Ca^{2+}, and vitamins may help in its prevention especially in pregnant and nursing women and children [234]. The mitigation of the genotoxic effects of fluoride and Al^{3+} was possible by ascorbic acid [102, 329, 330]. Treatments of vitamins C, D, and Ca^{2+} showed significant improvement in skeletal, clinical, and biochemical parameters in children consuming water containing 4.5 ppm of fluoride.

CLOSING COMMENTARY

Science has already accumulated evidence demonstrating how diverse molecules and biological processes can be affected by fluoride. It is conceivable that exposure of cells/tissues to fluoride could lead to a depletion of ATP, GTP, and PLC substrates. Fluoride intesifies LPO and protein oxidation and reduces the antioxidant potential in the cells. While fluoride in whole organism may not reach concentrations, which were used in the laboratory experiments *in vitro*, there may be instances where fluoride ions reach microenvironments where interference may occur, especially at the active sites of certain enzymes. Later it was demonstrated that AlFx causes many effects primarily attributed to fluoride. The discovery of synergistic action of fluoride plus Al^{3+} expanded our understanding of mechanisms of fluoride effects on living organism. The presence of AlFx has been demonstrated by many studies with crystallized proteins, intact cells, and whole animals.

The widespread use of fluoride as a general activator of heterotrimeric G proteins provided evidence that AlFx is a molecule giving false messages, which are amplified by processes of signal transduction. The phosphate analogue model of AlFx has been extended to many enzymes that bind phosphate groups. Regarding the role of phosphoryl transfer reactions in cell metabolism, we can predict hundreds of reactions, which might be influenced by AlFx. It seems probable that we shall not find any physiological process, which is not potentially influenced by synergistic action of fluoride plus Al^{3+}. The actual phosphorylation level of a given protein is the result of a delicate balance between kinases and phosphatases. Recent studies are highlighting the importance of tyrosine and serine/threonine phosphatases in the regulation of many different cellular processes. Fluoride in high concentrations (up to 100 mM) has been included into various design systems as a putative inhibitor of phosphatases. The potential interactions of fluoride in study design warrant a careful assessment and further investigation. The discoveries of receptor diversity, numerous isoforms of G proteins, and effector molecules broaden enormously the possibilities of interactions of signal transduction events. The use of fluoride in laboratory investigations contributed to the discovery of new signaling pathways and their cross talk. On the other hand, it is evident that fluoride might evoke disturbances of the communication networks.

The explanation of the observed effects in animals and humans is complicated by the chemical interactions of fluoride, Al^{3+}, and AlFx with numerous non-protein and protein ligands in body fluids and inside the cells. Competing reactions and disruptions of homeostasis can produce a hormetic dose-response and elicit unexpected responses. Also the fluoride effects on production of free radicals might have adverse influences on the defence mechanisms. It is therefore difficult to predict the actual effective concentrations of fluoride. It is evident that the definition of a "safe" concentration of fluoride for humans must consider that the dose, at which beneficial effects such as caries reduction are expected, is not far away from that one, which causes chronic, yet sub clinical toxic effects. The severity and the development of symptoms depend on age, nutrition status, kidney function, and many other factors.

Understanding the role of G proteins in cell signaling allow the hypothesis that the synergistic action of fluoride and Al^{3+} in the environment, water, and food chains, might impair many physiological functions of human body. The origins of many human diseases are in the malfunctioning of signaling components. Signaling disorders represent a major cause for the pathological states and many of the recently identified validated target molecules of drug research are signal transduction related macromolecules, mostly kinases [331]. Strunecka and Patocka [6] proposed that fluoride could complex with any pre-existing Al^{3+} within body fluids to produce the AlFx and this could lead to a combination of chronic activation of G protein regulated systems and suppression of other critical enzymes, especially kinases. Clinical, epidemiological, and ecological studies over the whole world bring evidence about potential health risks of chronic human exposure to fluoride and Al^{3+}.

One reason to suspect AlFx as the true culprit in these studies is that both Al^{3+} and fluoride is known to exist in appreciable concentrations in all commercial animal feeds and that Al^{3+} readily complexes with fluorine. The same is true with public drinking water. The water supply industry uses aluminum salts to produce a less turbid drinking water. Pesticides and fertilizers also increase fluoride content of food and processed beverages. The trend toward fluorinating pharmaceuticals increases fluoride exposure via medication. Al^{3+}, the metal of the earth's lithosphere, is everywhere: in water sources, in food chains, and in air in the form of dust particles. Contact of food and beverages with Al^{3+} during processing and storage can increase food levels of Al^{3+}. With exposure so common, we can no longer afford to ignore potential consequences of fluoride plus Al^{3+} for human health. The awareness of increasing load of fluoride and Al^{3+} as a new ecotoxicological phenomenon could contribute to the qualified assesment of their widespread use.

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ABBREVIATIONS

$[Ca^{2+}]_i$	=	Cytosolic Ca^{2+} level
Aβ	=	β-amyloid
AA	=	Arachidonic acid
AC	=	Adenylyl cyclase
AChE	=	Acetylcholinesterase
AD	=	ALZHEIMER'S disease
AlFx	=	Aluminofluoride complexes
AMPA	=	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ApoE4	=	ε4 allele of apolipoprotein E
ATPase	=	Adenosine triphosphatase
BuChE	=	Butyrylcholinesterase
cAMP	=	Cyclic AMP
cGMP	=	Cyclic GMP
CNS	=	Central nervous system
cPIP	=	Prostaglandylinositol cyclic phosphate
DAG	=	1,2-diacylglycerol
DF	=	Dental fluorosis
DHDG	=	*N*-(3-dodecyloxy-2-hydroxy propyl)-*N,N*-dimethylglycine
EC	=	Endothelial cells
ERK	=	Extracellular signal-regulated kinase
GABA	=	γ-amino butyric acid
GAP	=	GTPase activating protein
GDP	=	Guanosine diphosphate
GLAST	=	Glutamate aspartate transporter
GTP(S)	=	Guanosine-5'-*O*-3-[^{35}S](thio)triphosphate
IDD	=	Iodine deficiency disorders

IL = Interleukin
IMPase = Inositol monophosphatase
$Ins(1,4,5)P_3$ = Inositol 1,4,5-trisphosphate
InsPs = Inositol phosphates
JNK = c-jun-NH_2-terminal kinase
KGDHC = α-ketoglutarate dehydrogenase complex
LPO = Lipid peroxidation
MAPK = Mitogen activated protein kinase
MIP-1α = Macrophage inflammatory protein-1α
NMDA = *N*-methyl-D-aspartic acid
NO = Nitric oxide
PA = Phosphatidic acid
PAF = Plateled activating factor
PDE = Phosphodiesterase
PG = Prostaglandin
PI 3-K = Phosphatidylinositol 3-kinase
PIP_2 = Phosphatidylinositol 4,5-bisphosphate
PKC = Protein kinase C
PLA_2 = Phospholipase A_2
PLC = Phospholipase C
PLD = Phospholipase D
RBC = Red blood cells
RNS = Reactive nitrogen species
ROS = Reactive oxygen species
SF = Skeletal fluorosis
T_3 = Triiodothyronine
T_4 = Thyroxine
TGF-β = Transforming growth factor-β
TNF-α = Tumor necrosis factor-α
TSH = Thyroid stimulating hormone
TX = Thromboxane

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